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A CRITICAL ROLE FOR ZINC IN ETHANOL ACTION AT THE GLYCINE RECEPTOR

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A CRITICAL ROLE FOR ZINC IN ETHANOL ACTION AT THE GLYCINE RECEPTOR

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May, 2012

Dedication

To the millions of people suffering from alcohol abuse and dependence and to those who have loved and lost someone to alcoholism, I dedicate this to each of your journeys.

As you endure the challenges and suffer the consequences of this disease, we as scientists continue our quests to better understand the sites and mechanisms by which alcohol exerts its intoxicating effects in hopes that one day improved treatment options will be developed.

Acknowledgements

I would like to extend my gratitude and appreciation to a number of people without whom this endeavor would not have been possible. First and foremost, I am infinitely grateful for the time, guidance, expertise, and patience of my research advisor, Dr. R. Adron Harris. His support and belief in me, especially in light of a series of unexpected personal adversities in the latter stages of this process, have made the successful completion of this work possible. Also, for his unwavering commitment and high standards, though challenging and frustrating at different points, have forced me to grow and mature both as a scientist and as a person, and it is with is that I feel prepared and determined to succeed in the next stages of my career.

Also, I extend many thanks to the other members of my dissertation committee: Drs. James Trudell, Christine Duvauchelle, Richard Morrisett, and Andrea Gore. Their support, expertise, cooperation, flexibility, and letters of recommendation are greatly appreciated. In addition, Dr. Trudell's role as a collaborator and his willingness to travel to Austin (from Stanford) for my committee meetings and defense are truly invaluable, and it is with great pleasure and privilege that I look forward to many more future collaborative endeavors. When I began graduate school, Dr. Gore was the graduate advisor for the Pharmacology and Toxicology Program, but since then Dr. Duvauchelle has assumed this role, and their commitment and dedication to the better interests of the students are truly an asset to the program. I am fortunate to have benefited from them fulfilling this role.

Though not on my dissertation committee, the support and guidance of several other people affiliated with The University of Texas (past and present) have contributed greatly to my successes. First, Dr. S. John Mihic, with whom I began my work at The

Waggoner Center, has had a major influence in my career beginning with advising me in pursuit of my undergraduate honors thesis, and since then he has provided me with innumerable valuable insights and comic relief through regular conversations. In addition, it was under his auspice that I received much technical training at the lab bench as an undergraduate student working in his lab. Also, I would like to acknowledge the efforts, cooperation, and insights of Dr. Yuri Blednov. Without Yuri, the completion of the behavioral experiments in this project would not have been possible. For his commitment and willingness to help me, especially through the course of my medical adversities, I am truly grateful. Furthermore, I feel forever indebted to Dr. Deborah Stote because without her help and guidanice I may never have found The Waggoner Center, specifically Drs. Harris and Mihic, and had the opportunity to pursue my passions starting as an undergraduate student.

I am also extremely thankful and appreciative for those in my personal life. To my parents, Edward and Lisa, thank you for your unconditional love and support in allowing me to pursue my dreams. You have instilled in me the motivation and discipline to not be good, but rather great at all that I do. To my twin sister, Mandy and the future Dr. M. McCracken, thank for you experiencing many aspects of this journey with me. On a daily basis you motivate and challenge me to achieve more than I would if were in this field alone. I appreciate your support and the maturity and enthusiasm with which you have handled my dissertation defense occurring first. Your time is coming, and I know you will achieve great things. In addition, to my many friends (those particularly closest know who they are) thank you for the unique ways that you each enrich my life.

I am very appreciative to M. June and J. Virgil Waggoner whose generous monetary donation in light of a great tragedy helped to establish The Waggoner Center

for Alcohol and Addiction Research. The success of this Center would not be possible without many people who have also been extremely helpful to me in my graduate career. Particularly Debra James, whose knowledge, skills, and personality create an environment in which much can be accomplished and enjoyed. Thank you Debbie for all that you do. In addition, I would like to acknowledge the many current and past members of the Harris for their help and support.

A Critical Role for Zinc in Ethanol Action at the Glycine Receptor

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The University of Texas at Austin, 2012

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Ethanol is a widely used drug, yet an understanding of its sites and mechanisms of action remains incomplete. Among the protein targets of ethanol are glycine receptors (GlyRs). In addition to ethanol, zinc also modulates GlyR function. Although the individual effects of zinc and alcohols on GlyR function have been well studied, the combined effects of these agents have not been thoroughly examined. This project investigated the effects of zinc on alcohol action at the glycine receptor (GlyR). In Aim 1, the effects of zinc on ethanol modulation of GlyR function were tested and characterized in three GlyR α subunits (α1-3). Aim 2 explored a site of action for the augmenting effects of zinc on ethanol action at the GlyR. Mutant D80A GlyRs, which lack a zinc binding site (D80), were constructed and allowed us determine if this zinc binding site is important for the zinc/ethanol interactions that were observed in Aim 1. The effects of ethanol were reduced in mutant D80A GlyRs compared to wild type (WT). In addition, manipulating zinc levels in our buffers either by adding or chelating zinc did not change the magnitude of ethanol enhancement of mutant D80A GlyRs as it did in WT GlyRs suggesting that the D80 position is important for zinc modulation of ethanol action. Finally, Aim 3 extended the findings from Aims 1 and 2 by evaluating the effects of a GlyR point-mutation on alcohol consumption and other behavioral tests in mice. *Glra1*(D80A) knock-in mice provided an animal model for behavioral studies of

zinc/ethanol interactions at the GlyR and showed decreased alcohol consumption and preference compared to their WT littermates. In addition, D80A KI mice had increased startle responses compared to their WT littermates. Other behavioral tests were also conducted including tests of ethanol motor incoordination and strychnine induced convulsions; there were no differences detected between KI and WT mice in these assays. Overall, our findings demonstrate that zinc is critical in determining the effects of ethanol at GlyRs and suggest that zinc signaling at the D80 position may be important for mediating the behavioral effects of ethanol action at GlyRs.

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CHAPTER I: Background and Significance

Human consumption of alcohol for its intoxicating effects has been a practice of people since prehistoric times, yet the complex biochemical actions of this drug that produce its physiological effects remain poorly understood today. As a sedative hypnotic agent, alcohol produces a progressive series of physiological and subjective behavioral changes that occur with increasing blood alcohol concentrations. At low blood alcohol concentrations, intoxicated individuals may experience reductions in anxiety, disinhibition, and 'social lubrication' (increased talkativeness, assertiveness, and eloquence), whereas at moderate blood alcohol concentrations, impairments in judgment, cognition, and motor function may be evident in addition to signs of marked ataxia, impaired reaction time, and blackouts. Finally, at high blood alcohol concentrations, individuals demonstrate increased sedation/hypnosis, and approach anesthesia and coma. The LD50 (or lethal dose for 50% of people) for alcohol is about 0.40 gm% (Koob and Le Moal, 2006). For comparison, the legal level for intoxication in the United States is 0.08 gm%.

Affecting all parts of the brain, alcohol specifically impacts coordination via action in the cerebellum, activates the mesolimbic dopamine system resulting in reward, and influences the function of cortical regions involved in impulsivity and decision-making (Roberts and Koob, 1997). The acute effects of alcohol are reversible in contrast to the persistent effects of chronic alcohol exposure. Repeated alcohol use can result in the development of alcohol tolerance, and periods of abstinence following chronic use can result in withdrawal symptoms,

which are two of the hallmark symptoms of alcoholism. Alcohol craving and a loss of control or inability to stop drinking are additional symptoms of this chronic, relapsing, and cureless disease. A number of other known alcohol-related toxicities and medical complications can occur with alcohol use, such as sexual dysfunctions, amenorrhea, liver damage/cirrhosis, heart disease, cancer, neuropsychiatric disorders (i.e. Wernicke's encephalopathy and Korsakaff's psychosis), and fetal alcohol syndrome (for review, Koob and Le Moal, 2006).

Although ethanol is among the most widely used drugs, our understanding of the molecular targets to which it binds to produce its intoxicating effects remains incomplete. One widely accepted idea is that ethanol acts at specific membrane proteins in the central nervous system. In a recent review, Harris et al. (2008) outline a set of criteria that qualifies a substrate as a molecular target of alcohol. Among the strongly-supported protein targets are glycine receptors (GlyRs), which mediate the majority of inhibitory neurotransmission in the brain stem and spinal cord (Legendre, 2001), but are also expressed in the hippocampus (Fatima-Shad and Barry, 1993), nucleus accumbens (Martin and Siggins, 2002; Molander and Soderpalm, 2005), amygdala (Delaney et al., 2010), olfactory bulb (van den Pol and Gorces, 1988), and cerebellum (Takahashi et al., 1992). In addition to ethanol (and other alcohols), several other pharmacologically-active agents, including volatile anesthetics and inhaled drugs of abuse, enhance GlyR function via allosteric modulation (Mihic et al., 1997; Beckstead et al., 2000). Zinc, which exists endogenously in brain both in protein-bound and free forms, also allosterically modulates GlyR function but does so in a biphasic manner such that concentrations less than 10 μM produce enhancing effects, and those greater

than 10 μM inhibit GlyR function (Bloomenthal et al., 1994; Harvey et al., 1999; Laube et al., 2000). Although the individual effects of zinc and alcohols on GlyR function have been well studied, the combined effects of these agents have not been thoroughly examined such that previous investigations have largely neglected the potential role of endogenous agents such as zinc in ethanol receptor pharmacology.

GLYCINE RECEPTORS AND ALCOHOL

GlyRs are anion-conducting members of the nicotinic acetylcholine superfamily of ligand-gated ion channels. Four α subunits (1-4), with 80-90% sequence identity among subunits, and a single β subunit, possessing ~50% sequence identity with the α subunits, have been identified (Lynch, 2004). GlyR subunits can assemble to form either homomeric or heteromeric pentamers containing an integral ion pore. Each subunit contains extracelluar N- and C-terminal regions, a large intracellular loop, and four alpha helical segments (TM1-4), which collectively constitute a transmembrane domain (Figure 1.1).

Cysteine substitution experiments in the α1 GlyR at serine-267 (S267) in TM2 and alanine-288 (A288) in TM3, involving covalent thiol binding or cross-linking, suggest an alcohol and anesthetic binding pocket within the transmembrane domain of each subunit (Mascia et al., 2000; Lobo et al., 2006; 2008). Specifically, S267 and A288 were initially shown to be essential in GlyR enhancement by these agents (Mihic et al., 1997), and more recently it has been shown that mutation of the neighboring positions Q266 and M287 also eliminates potentiation of GlyR function by ethanol (Borghese et al., 2012). Furthermore,

findings from a series of studies indicate that amino acid residues in the TM1 and TM4 helices may also participate in alcohol and anesthetic binding (Lobo et al., 2004; 2006; 2008; McCracken et al., 2010).

Additional putative alcohol binding sites on the α1 GlyR have also been suggested. These include alanine-52 (A52), which is in Loop 2 of the N-terminal domain (Davies et al., 2004; Crawford et al., 2008), as well as lysine-385 (K385) of the large intracellular loop linking TM3 and TM4 (Yvenes et al., 2008). The latter residue has also been suggested to be involved in GlyR modulation by Gβγ (Yvenes et al., 2003).

Although less sensitive to the effects of alcohol than the α1 subunit, α2 GlyR function is also enhanced by ethanol, and of the GlyR α subunits it predominates in limbic brain regions affected by ethanol (McCool et al., 2003). The differences in alcohol sensitivity between the α1 and α2 subunits may reflect the presence of non-homologous residues in the N-terminal domain. For example, α1A52S GlyRs resemble wild type α2 receptors in their sensitivity to ethanol (i.e. the α1 A52S mutant is less sensitive than α1 wild type) (Mascia et al., 1996). Because sensitivity to the effects of alcohols has not been published for α3 and α4 subunits, comparisons of the relative magnitude of the effects of ethanol on these subunits to either α1 or α2 are difficult to make at this time.

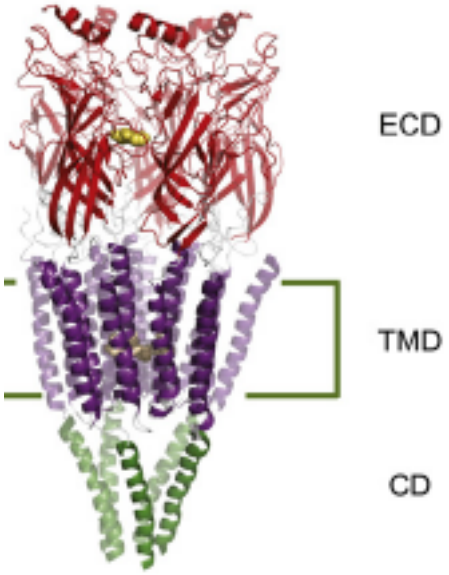

Figure 1.1: 3D structure of a pentameric ligand-gated ion channel.
The extra-cellular domain (ECD) is shown in red, the transmembrane domain (TMD) is depicted in purple, and the intra-cellular loop or cytoplasmic domain (CD) is displayed in green. Because X-ray crystal structures have not been solved for GlyRs, homology models of GlyR structure are generated using related proteins of known structure as templates. This illustration is based on the known structure of the Torpedo nicotinic acetylcholine receptor (adapted from Baenziger & Corringer, 2011).

Recent single channel analysis of ethanol modulation of GlyR function has revealed that ethanol potentiates receptor function by increasing burst durations and the number channel openings per burst. In addition, kinetic modeling suggests this is likely due to ethanol decreasing glycine unbinding from the receptor (Welsh et al., 2009).

Behavioral studies in rodents have revealed a possible role for GlyRs in alcohol consumption. For example, microdialysis of glycine into the nucleus accumbens increases extracellular accumbal dopamine levels, and this is accompanied by a decrease in alcohol consumption by alcohol-preferring Wistar rats; in contrast, the GlyR antagonist strychnine produces opposite effects (Molander et al., 2005). Strychnine, applied via microdialysis, prevents increases in accumbal dopamine levels after either local or systemic alcohol administration (Molander and Soderpalm, 2005). Furthermore, in line with these findings, the glycine reuptake inhibitor, Org 25935, has been shown to decrease ethanol, but not water intake, as well as ethanol preference (Molander et al., 2007).

GLYCINE RECEPTORS AND ZINC

Although zinc concentrations in the brain exceed those present in other organs, most zinc is protein-bound (Mathie et al. 2006). In its free or rapidly exchangeable form, zinc exists in cerebospinal fluid at tonic baseline concentrations ranging from approximately 5-25 nM (Frederickson et al. 2006). Present in higher concentrations in the grey than in the white matter, zinc is most abundant in forebrain areas including the hippocampus, amygdala, and neocortex (Slomianka et al., 1990; Frederickson & Moncrieff, 1994; Takeda et al., 2000). In

the CNS, neurons secrete zinc (Takeda et al., 2001), which is predicted to remain at concentrations up to 10 μM following presynaptic release from GABAergic, glutamatergic, or glycinergic terminals (Frederickson et al. 2001). In its free and loosely bound forms zinc has been localized in synaptic vesicles containing zinc transporters, ZnT3 and ZnT4, which are required for its neuronal transport (Harris, 2002). Vesicular zinc is often found in GABAergic or glutamatergic neurons, and the localization of zinc in the terminals of these neurons follows regional patterns. Specifically, zinc-enriched GABAergic terminals are found in the cerebellum (Wang et al. 2002), whereas glutamatergic terminals containing vesicular zinc predominate in the cerebral cortex, amygdalar nuclei, olfactory bulb, and the hippocampal formation (Frederickson & Bush, 2001), which notably also contain the GlyR subunits α1, α2, and α3. In addition to brain, glycine and zinc have been co-localized at synapses in the spinal cord (Birinyi et al., 2001).

Although the mechanism for zinc modulation of GlyR function is not completely understood, several amino acid residues located in the *N*-terminal domain of the α1 subunit responsible for the enhancing and inhibiting effects of zinc on GlyR function have been identified (Table 1). The potentiating effects of zinc, generally seen at concentrations in the nanomolar to low micromolar range (<10 μM), require high-affinity binding to amino acids at positions aspartate-80 (D80), threonine-151 (T151), glutamate-192 (E192), aspartate-194 (D194), and histidine-215 (H215) (Laube et al. 2000; Miller et al. 2005b). Additional residues of the GlyR α1 subunit, in particular histidine-107 (H107), histidine-109 (H109), threonine-112 (T112), and threonine-133 (T133) are thought to contribute to lower-affinity binding sites and are necessary for inhibition of GlyR function by

higher micromolar concentrations of zinc (> 10 μM) (Harvey et al., 1999; Laube et al., 2000; Miller et al., 2005). The importance of zinc binding at lower affinity (GlyR inhibitory) sites is illustrated in instances of ischemia, seizure, trauma, and neurodegeneration, during which zinc levels are estimated to peak in the brain at concentrations in excess of 100 μM (Choi & Koh,1998; Doraiswamy & Finefrock, 2004).

Alignment of the amino acid sequences for the GlyR α1, α2, and α3 subunits reveal that while most of the positions that have been demonstrated to be important for GlyR modulation by zinc are conserved across all three subunits, there are positions that are not (see Table 1). For example, all of the putative zinc binding positions in the α1 GlyR are conserved at the homologous positions in the α2 and α3 subunits except for H107 in α1, which is asparagine-114 (N114) and asparagine-107 (N107) in α2 and α3 respectively. The significance of this difference can be seen such that homomeric α1 GlyRs are more sensitive to zinc inhibition than homomeric α2 or α3 GlyRs, but mutation of histidine-107 to asparagine results in an α1 H107N mutant that is less sensitive to zinc inhibition resembling α2 and α3 (Miller et al., 2005). In addition, D194 in α1 and α3, is not conserved at the homologous position in α2, which is glutamate-201 (E201). Studies of the enhancing effects of zinc on GlyRs have revealed the potential importance of this site in zinc binding and action. Homomeric α2 GlyRs are less sensitive to the potentiating effects of zinc than are homomeric α1 or α3 GlyRs. However, mutation of E-201 to aspartate (E201D) in α2 results in a mutant GlyR that is more sensitive to zinc enhancement, and thus resembles wild type α1 and α3 GlyRs (Miller et al., 2005).

α1	D80	H107*	H109	T112	T133	T151	E192	D194	H215
α2	D87	N114	H116	T119	T140	T158	E199	E201*	H222
α3	D80	N107	H109	T112	T133	T151	E192	D194	H215

Table 1: Zinc binding positions on GlyR subunits.
Alignment of the amino acid sequences for the α1, α2, and α3 GlyR subunits reveal that of the known putative zinc binding sites at the GlyR all are conserved in these three subunits except for two positions (*), H107 in α1 and E201 in α2. Amino acid residues displayed in green are important for high-affinity zinc binding and enhancement of GlyR function, whereas those denoted in red are critical for low-affinity zinc binding and inhibition of GlyR function.

Several lines of evidence highlight the physiological relevance of free zinc in the CNS and its ability to modulate GlyR function there. For example, the significance of zinc to normal GlyR function was recently demonstrated in knockin mice carrying a D80A mutation in the GlyR α1 subunit gene (Glra1) (Hirzel et al., 2006). Mice homozygous for Glra1 (D80A) exhibit phenotypes analogous to human startle disease, and in vitro studies of spinal neurons and brainstem slices from these animals reveal significant impairments in the enhancement of spontaneous glycinergic currents by zinc (Hirzel et al., 2006). In addition, basal concentrations (low nanomolar) of zinc found in the CNS are sufficient for the prolongation of the decay phase of glycinergic miniature inhibitory postsynaptic currents (Suwa et al., 2001).

Single channel kinetic analysis of potentiation of GlyR function by low concentrations of zinc have shown that like ethanol zinc enhances GlyR function by increasing the frequency of channel opening and increasing burst durations (Laube et al., 2000).

Although the individual effects of zinc and alcohols as modulators of GlyR function have been well studied, the combined effects of these agents have not been thoroughly examined such that most studies have largely neglected the potential role of endogenous agents such as zinc in ethanol receptor pharmacology.

However, emergent evidence from *in vitro* investigations in the α1 subunit suggest that zinc is critical for ethanol enhancement of GlyR function by intoxicating concentrations of ethanol.

Specifically, McCracken et al. (2010) demonstrated that low nanomolar concentrations of zinc both enhanced responses to submaximal concentrations of glycine and increased ethanol enhancement of GlyR function (Figure 1.2). In addition, chelation of free zinc by tricine decreased the potency of submaximal concentrations of glycine and reduced the magnitude of ethanol's effect on GlyR function indicating the importance of zinc in determining the sensitivity of GlyRs to ethanol (Figure 1.3). Subsequently, careful pharmacological characterization of a GlyR α1 mutant (M287L) with decreased ethanol sensitivity revealed that although enhancement of the mutant by zinc alone was retained, zinc did not enhance the effects of ethanol on the mutant receptor at low nanomolar concentrations that were suffice for increasing the magnitude of ethanol enhancement of receptor function in wild type GlyRs (Borghese et al., 2012).

GLYCINE RECEPTOR MUTANT MICE

Receptor knock-in (KI) mice, containing a single point mutation, serve as a powerful tool to examine the effects of ethanol on the CNS. In such mice, all other aspects of the receptor of interest function normally, which diminishes the likelihood of compensation by other receptor types and subunits. To date, multiple behavioral pharmacological studies of ethanol action in GlyR mutant mice have been conducted, but have been limited to mice carrying mutations in the GlyR α1 subunit gene (*Glra1)* that correspond to single amino acid substitutions (S267Q, Q266I, and M287L) in the alpha helical segments of the transmembrane domain. In addition, homozygous KI mice carrying each of the *Glra1* S267Q, Q266I, and M287L mutations are not viable, and therefore all

ethanol-related behavioral tests of these mutations have been additionally limited to heterozygous animals (Findlay et al., 2003; Blednov et al., 2012). Despite most homozygous GlyR KI mice not being viable, one exception is the *Glra1* D80A KI animal, which contains a point mutation at a putative high affinity zinc binding site located in the N-terminal domain.

Preliminary non-ethanol related behavioral characterizations of homozygous *Glra1* D80A mice reveal that enhancement of GlyR function by endogenous zinc is critical for normal neurological function (Hirzel et al., 2006). The availability of D80A mice affords the opportunity to study the potential role of zinc signaling at GlyRs in modulating alcohol drinking and other alcohol-related behaviors.

In an alternative approach, transgenic expression of α1 S267Q GlyRs in mice under the control of synapsin I regulatory sequences have been developed and studied in alcohol-related behavioral tests. Although these mice have been helpful in demonstrating the importance of normal glycine receptor function in many alcohol-related behaviors, the transgenic approach is overall inferior to the KI approach in that the latter uses endogenous promoters to drive expression, which therefore more closely resembles wild type cellular distributions.

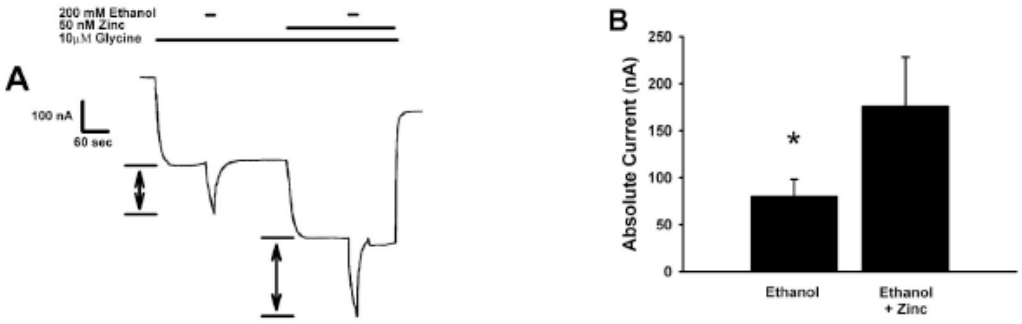

Figure 1.2: The effects of added zinc on ethanol action at the glycine receptor. A) 200 mM ethanol was first co-applied with 10 μM glycine and then was reapplied after a washout period with both 10 μM glycine and 50 nM zinc. B) The amount of absolute current produced by 200 mM ethanol was significantly greater when it was applied during a concurrent application of 50 nM zinc + 10 μM glycine than when it was applied with 10 μM glycine alone (adapted from McCracken et al., 2010).

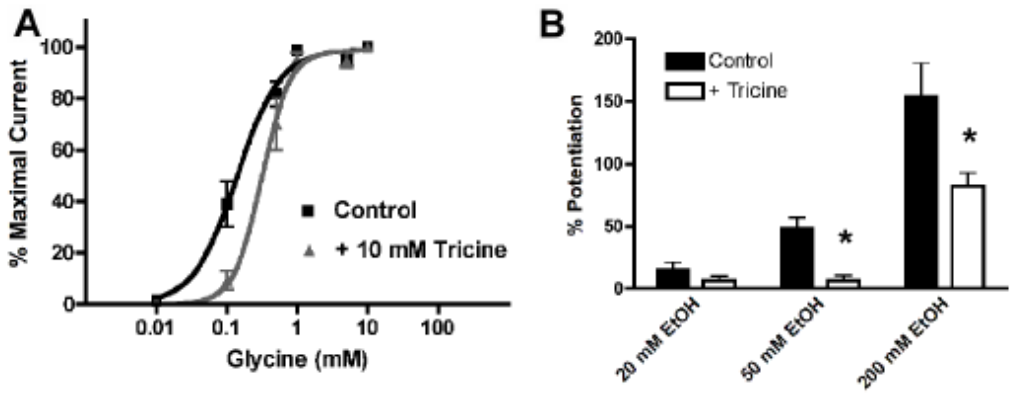

Figure 1.3: The effects of zinc chelation on Glycine receptor sensitivity to glycine and ethanol.
Glycine concentration-response curves and EtOH enhancement of EC5-10 glycine responses were determined before and after application of 10 mM tricine. A) Tricine did not affect the currents elicited by a maximally-effective concentration of glycine (10 mM) although it did increase the glycine EC50 from 139 μM to 315 μM; i.e., it only decreased the effects of submaximal glycine concentrations. B) Tricine significantly decreased the degree of GlyR enhancement by 50 mM and 200 mM ethanol (adapted from McCracken et al., 2010).

ALCOHOL-RELATED BEHAVIORAL TESTING OF MUTANT MICE

Several behavioral paradigms have been developed, adapted, and validated for testing alcohol consumption and other ethanol-related behaviors in rodent animal models. For example, the 24-hour access two-bottle choice alcohol drinking paradigm, which was developed several decades ago, has been used in innumerable tests aimed at identifying genetic determinants of, as well as the effects of voluntary ethanol consumption in rodents (Crabbe et al., 1984).

In addition to tests of voluntary alcohol drinking, several other behavioral measures of ethanol action have specifically been used in characterizing the behavioral phenotypes of GlyR mutant mice. For example, duration of LORR has been often used in tests of sensitivity to ethanol and other sedative agents including in studies of mice with GlyR mutations (Findlay et al., 2005; Blednov et al., 2012). Furthermore, acoustic startle responses have been characterized in both homozygous and heterozygous GlyR KI mice with and without ethanol (Findlay et al., 2003; Hirzel et al., 2006; Blednov et al., 2012). Because strychnine is a known antagonist of GlyR function, measurements of strychnine-induced convulsions in mice containing GlyR mutations are important in determining the behavioral effects of such mutations. Previous studies of mice with GlyR mutations have not only measured strychnine-induced seizure activity, but some also have measured ethanol inhibition of strychnine convulsions (Findlay et al., 2002; Blednov et al., 2012).

GAPS IN OUR KNOWLEDGE

Despite the contributions of studies that have investigated the effects of zinc and/or ethanol on GlyR function, several unknowns still exist. First, initial findings from McCracken et al. (2010) suggest that zinc enhances the effects of ethanol at GlyRs, however, it is unknown whether zinc enhancement of ethanol action at GlyRs is due to zinc binding and action at known zinc binding sites or whether this effect is due to zinc action at novel positions. *In vitro* investigations of the effects of ethanol on α1 D80A GlyRs provide a reasonable starting point for such studies as this position is among the N-terminal domain sites known to be important for the enhancement of GlyR function by zinc. In addition, because *Glra1* D80A KI mice are viable, they provide an animal model for alcohol-related behavioral studies of the effects of zinc signaling at GlyRs. Such knowledge is essential for understanding how ethanol enhances GlyR function, and this more broadly could provide more accurate approximations of ethanol's effects *in vivo*. In addition, little work has focused on the GlyR α3 subunit, and unlike the α1 or α2 subunits, the effects of ethanol on the α3 subunit, which is distributed in nociceptive neurons of the spinal cord dorsal horn (Harvey et al., 2004), the amygdala (Delaney et al., 2010), the nucleus accumbens (Jonsson et al., 2009), the hippocampus (Meier et al., 2005), the retina (Haverkamp et al., 2003) as well as other CNS systems, remain unknown at this time. This pattern of expression of the α3 GlyR subunit in the CNS suggests it may be important in motivation, learning, memory, and other cognitive processes, which are impacted by exposure to ethanol, and thus warrants inclusion in studies of alcohol action. This dissertation contains three aims that each contribute to the overall goal of

providing a more thorough understanding of ethanol's action at GlyRs. Aim 1 characterizes zinc/ethanol interactions at three GlyR subunits (α1, α2, α3), and Aim 2 explores a site of action for such an interaction using GlyRs containing mutations at putative zinc binding sites. Finally, Aim 3 investigates the behavioral effects of ethanol in KI mice containing GlyR mutations at a putative zinc binding site (D80A).

CHAPTER II: Zinc-dependent Modulation of α2 and α3 Glycine Receptor Subunits by Ethanol

INTRODUCTION

Although ethanol is among the most prevalently used drugs, the sites and mechanisms of action by which it produces its intoxicating effects are not thoroughly understood. One widely accepted idea is that ethanol acts at protein targets in the body. Among the strongly- supported protein targets of ethanol are strychnine-sensitive glycine receptors (GlyRs) (Harris et al., 2008), which belong to the Cys-loop superfamily of ligand-gated ion channels. Like other members of this channel family that also includes nicotinic acetylcholine receptors, GABA-A receptors, as well as serotonin-3 receptors, GlyRs are pentameric proteins containing an integral channel pore.

There are four known GlyR alpha subunits (α1, α2, α3, and α4) and one known GlyR beta subunit (Lynch et al., 2004). GlyRs can assemble to form either homomeric or heteromeric chloride channels that mediate inhibitory neurotransmission in the central nervous system where they are localized most abundantly in the spinal cord and brainstem (Legendre et al., 2001), but are also expressed appreciably throughout the brain in many regions including the cortex, hippocampus, amygdala, nucleus accumbens, striatum, ventral tegmental area, and cerebellum (Takahashi et al., 1992; Jonsson et al., 2009; Baer et al., 2009).

Identified as one of the strongly-supported molecular substrates of ethanol, GlyRs are not only modulated by alcohols, but also by volatile anesthetics, and inhaled drugs of abuse, which all act to enhance receptor function (Mihic et al., 1997; Beckstead et al 2002; Harris et al., 2008). In addition to these exogenous

agents, endogenous cations such as zinc, also allosterically modulate GlyR function. Unlike the exogenous sedative hypnotics that strictly enhance GlyR function, zinc produces biphasic effects on GlyR function such that nanomloar and low micromolar concentrations enhance glycine-activated currents, whereas higher micromolar concentrations of zinc inhibit GlyR function (Bloomenthal et al., 1994; Laube et al., 2000; Miller et al., 2005). In addition, recent *in vitro* work in the GlyR α1 subunit has shown that zinc at physiological (nanomolar) concentrations enhances the magnitude of ethanol's effects on GlyR function (McCracken et al., 2010).

Although the effects of ethanol on the α1 GlyR subunit have been relatively well studied, much less work has focused on the GlyR α2 subunit, and presently there are no published reports of ethanol modulation of the GlyR α3 subunit despite that both α2 and α3 GlyR subunits are expressed in a number of brain regions involved in ethanol consumption and other ethanol-related behaviors (Jonsson et al., 2009). More specifically, in limbic and motivation centers of the brain, such as the amygdala and nucleus accumbens, studies of gene and receptor membrane expression demonstrate that there is greater abundance of α2 and α3 than α1 subunits in these regions suggesting the importance of studies to better characterize the effects of ethanol on these particular GlyRs (Delaney et al., 2010; Jonsson et al., 2009).

In addition to differences in brain region expression among GlyR alpha subunits, there is emergent evidence suggesting some functional differences as well. For example, subpopulations particularly of α3 subunits undergo RNA editing that results in α3P185L receptors. These edited subunits confer high

sensitivity to agonists such as glycine (Meier et al., 2005; Legendre et al., 2009), which seems to be functionally important for tonic inhibition (Eichler et al., 2009). Recent studies of RNA editing largely focus on α3 GlyRs, but there is some evidence for a similar post-translational modification in α2 GlyRs as well (Eichler et al., 2008).

Due to their localization in limbic, motivation, and reward-related brain areas, thorough studies of the effects of ethanol at α2 and α3 GlyRs are necessary and relevant for better understanding the mechanism by which ethanol exerts its physiological effects. In addition, because zinc exists is the CNS at tonic baseline levels in the low nanomolar range (Frederickson et al., 2006), which is sufficient for enhancement of GlyR function and critical for determining the magnitude of ethanol's effects on α1 GlyRs, it is important to investigate the role of zinc in ethanol modulation of α2 and α3 GlyR subunits. Furthermore, emergent information about the significance of RNA edited GlyRs in CNS inhibition suggests that comparisons of ethanol sensitivity between edited and unedited receptors may be important for a thorough understanding of ethanol action.

In the current study, we tested the hypothesis that the magnitude of ethanol's effects on α2 and α3 GlyR subunits like α1 subunits would be enhanced in the presence of physiologically relevant concentrations of zinc. Because the effects of ethanol on α3 GlyRs have not been previously studied, sensitivity to ethanol had to be first confirmed in this subunit. A secondary goal of this study was to subsequently determine whether or not there are differences in ethanol sensitivity between edited and unedited α3 GlyRs.

MATERIALS AND METHODS

Materials

All chemical reagents (tricine, zinc chloride, ethanol, glycine) and Modified Barth's Solution (MBS) buffer constituents (NaCl, KCl, NaHCO3, HEPES, MgSO4) were purchased from Sigma-Aldrich (St. Louis, MO). *Xenopus laevis* frogs were purchased from Xenopus Express (Brooksville, FL).

cDNA Constructs and Site-directed Mutagenesis

Glycine receptor α2 (human) and α3 (rat) subunit clones (both in pCis2 vectors) were obtained from Dr. N. Harrison and Dr. H. Betz, respectively. A mutant α3 GlyR was created by substituting the proline at position 185 with leucine in order to create an α3P185L mutant GlyR that corresponds to RNA edited GlyRs occurring *in vivo*. The mutagenesis reaction was carried out using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and commercially engineered mutagenic primers (Integrated DNA Technologies, San Diego, CA). Successful mutagenesis was confirmed using DNA sequencing at The University of Texas DNA Core Facility (Austin, TX).

Xenopus Oocyte Preparation

Partial ovariectomies were performed on sexually mature female *Xenopus laevis,* and ovary fragments were placed in isolation media (108 mM NaCl, 2 mM KCl, 1 mM EDTA, 10 mM HEPES). Stage IV and V oocytes were manually extracted from the thecal and epithelial membranes with forceps under a light microscope. In order to remove the follicular membrane, isolated oocytes were immersed in 0.5 mg/ml collagenase in collagenase buffer (83 mM NaCl, 2 mM

KCl, 1 mM $MgCl_2$) for 10 minutes and then were transferred into MBS (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$) for cDNA injection.

The animal pole of each oocyte was injected with (1.5 ng / 30 nl) either α2, wild type α3, or mutant α3P185L GlyR cDNA using an automated injector interfaced to a foot switch (Drummond Nanoinject II, Broomall, NY). Injected oocytes were incubated individually in 96-well plates filled with incubation media (MBS, 2 mM Na pyruvate, 0.5 mM theophylline, 10 U/ml penicillin, 10 mg/l streptomycin, 50 mg/l gentamycin) and were stored in the dark at 13C until electrophysiology recordings were performed approximately 1-7 days following injection.

Two-Electrode Voltage Clamp Electrophysiology

For electrophysiology recordings, oocytes were impaled in the animal poles with two high-resistance (>1 MΩ) glass electrodes containing 3N KCl and were voltage-clamped at -70mV using a Warner OC-725C oocyte clamp (Warner Instruments, Hamden, CT). A Masterflex USA peristalsis pump (Cole-Parmer Instrument Corporation, Vernon Hills, IL) was used to deliver MBS to oocytes via bath perfusion at a rate of 2 ml/min. Clamping currents were recorded on LabChart Pro software (Colorado Springs, CO), which was interfaced to the oocyte voltage-clamp apparatus via a PowerLab 4/30 data acquisition system (AD Instruments, Colorado Springs, CO).

Glycine concentration-response curves. Glycine concentration response curves were generated for oocytes expressing α2 and α3 GlyRs. For wild type

receptors, a series of glycine concentrations (10 μM- 100 mM) were tested. Each respective concentration of glycine was applied for ~30 sec and a 7 min washout period followed each application. The concentration of glycine that elicited the largest response was determined to be maximal, and the effects of the remaining glycine concentrations were calculated and recorded as a percent of the maximal glycinergic effect. To determine the effects of zinc chelation on sensitivity to glycine, the same protocol was repeated in a buffer solution containing 10 mM tricine. Similarly, to determine the effects of added zinc on glycine responses, the same glycine response curve protocol was again repeated, but in the presence of 1 μM zinc added to the buffer solution. Because α3P185L GlyRs are known to confer very high sensitivity to glycine (Legendre et al.,), this difference in glycine sensitivity between wild type α3 and α3P185L GlyRs was confirmed by generating concentration-response curves as described above. However, a wider range of glycine concentrations was tested (100 nM- 100 mM) due to published reports of edited α3 GlyRs conferring significantly higher affinity for glycine than wild type channels (Meier et al., 2005; Legendre et al., 2009).

Ethanol sensitivity in the absence and presence of zinc. Oocytes were first perfused with two ~15 sec applications of 10 mM glycine each followed by a 7 min washout period. The peak current elicited by the second application of glycine was considered to be the maximal response and was used to determine a concentration of glycine that produced ~5-10% (EC5-10) of the maximal glcyinergic effect. This experimentally derived EC5-10 concentration of glyince was then applied and following a 7 min washout period, ethanol was first applied alone for 1 min, then it was applied concurrently with the experimentally derived

EC5-10 concentration of glycine for ~45 sec. The EC5-10 concentration of glycine was again applied alone for 45 sec following a 7 min washout. This procedure was used to test the effects of a series of ethanol concentrations (20, 50, 100, and 200 mM). To determine the effects of chelating contaminating zinc on ethanol modulation of α2 and α3 GlyRs, we repeated the same protocol in the presence of 10 mM Tricine, which was added to the buffer solution (as previously described in McCracken et al., 2010). In addition, to test the hypothesis that added zinc would produce the opposite effect of a zinc chelator, we repeated the procedure for testing ethanol sensitivity a third time in a buffer solution containing 500 nM added zinc. In all three conditions, the effects of ethanol were determined as percent potentiation of the glycine EC5-10 response.

Ethanol sensitivity in edited α3 GlyRs. To preliminarily screen for potential differences in ethanol sensitivity between edited and unedited α3 GlyRs, the above protocol was used to test the effects of 20 mM, 50 mM, and 200 mM ethanol on wild type α3 and mutant α3P185L GlyRs in standard MBS buffer solution.

DATA ANALYSIS

Nonlinear regression analyses were performed to calculate glycine EC 50 values and Hill coefficients for glycine concentration-response curves, and one-way ANOVAs followed by Tukey's post-hoc analyses were used to determined differences in ethanol sensitivity. Overall, statistical differences were determined at $p < 0.05$, and all analyses were conducted using GraphPad PRISM software (San Diego, CA).

RESULTS

For wild type α2 and α3 GlyRs, manipulating zinc levels shifted the respective glycine concentration-response curves of both subunits. More specifically, Figure 2.1 shows that adding 1 μM zinc, which is an approximately maximally enhancing concentration of zinc (Bloomenthal et al., 1994), resulted in leftward shifts, whereas chelating contaminating zinc with tricine resulted in slight rightward shifts. In addition, neither adding nor removing zinc from our MBS buffers changed the maximal glycine-activated currents for either α2 or α3 GlyRs.

Recently, we reported that the effects of ethanol on wild type α1 GlyRs are zinc dependent (McCracken et al., 2010), and in this study we sought to test the hypothesis that zinc is also critical in determining the magnitude of ethanol's effects on α2 and α3 GlyRs. First, we tested the effects of ethanol (20, 50, 100, and 200 mM) on these GlyRs in standard MBS and then subsequently in the presence of the zinc chelating agent tricine as well as in the presence of 500 nM added zinc. Figure 2.2 shows that chelating contaminating zinc with tricine decreased ethanol enhancement of α2 GlyR function. In contrast, the magnitude of the effects of ethanol was significantly greater in the presence of 500 nM added zinc [$F(6, 45)= 3.76$; 0.0041]. Because there are no published reports of ethanol modulation of α3 GlyRs, we first tested the hypothesis that the α3 GlyR subunit like the α1 and α2 subunits is sensitive to the effects of ethanol. Figure 2.3 shows that α3 GlyRs confer concentration-dependent sensitivity to a range of ethanol concentrations (20- 200 mM).

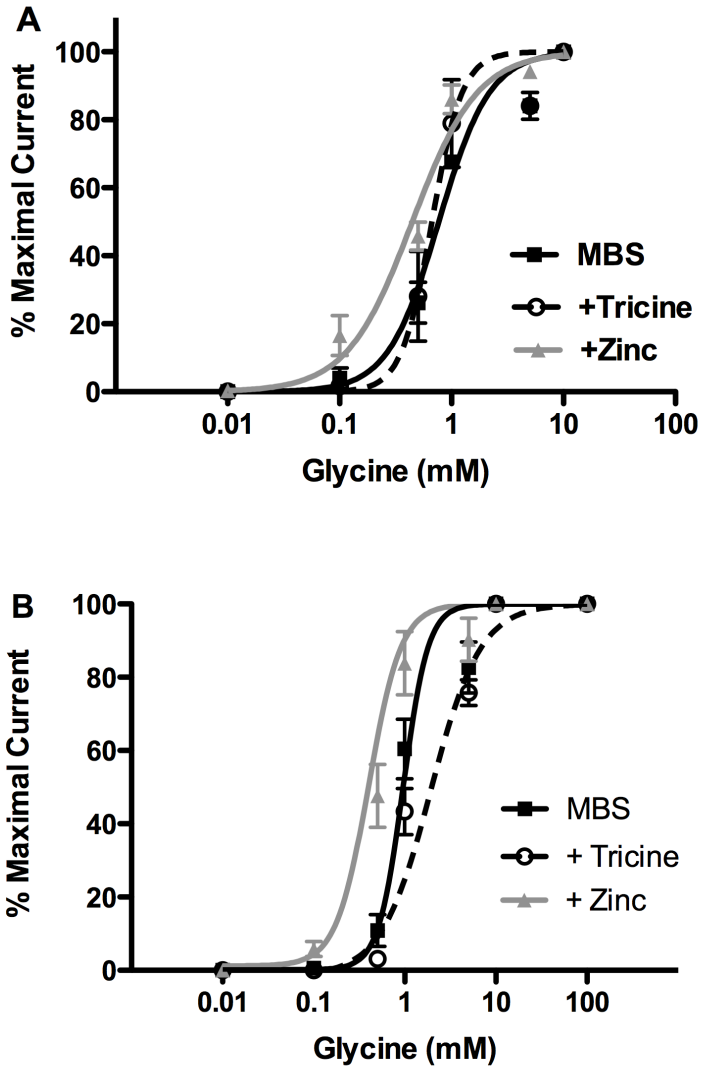

Figure 2.1. Glycine Responses in the presence of tricine.
Glycine concentration- response curves were generated for α2 and α3 GlyRs. A) The responses of α2 GlyRs to a series of glycine concentrations (10 μM-10 mM) in standard MBS, MBS with 10 mM trcine, and MBS with 1 μM added zinc. B) The responses of α3 GlyRs to glycine (10 μM-100 mM) in MBS, MBS with 10 mM tricine, and MBS with 1 μM added zinc.

After confirming ethanol enhancement of α3 GlyRs, we subsequently investigated whether or not the effects of ethanol are modulated by zinc as they are in α1 and α2 GlyRs. Chelating contaminating zinc resulted in decreased effects of ethanol, whereas adding 500 nM zinc increased the magnitude of ethanol enhancement of α3 GlyR function [$F(6,33)= 4.51$; $p= 0.0019$] (Figure 2.3). Recent findings indicate that some α3 GlyRs undergo RNA editing resulting in α3P185L GlyRs with high agonist sensitivity (Meier et al., 2005; Legendre et al., 2009). Our goal was to test for differences in ethanol sensitivity between wild type α3 GlyRs and α3P185L GlyRs containing a proline to leucine substitution that corresponds to RNA editing α3 GlyRs occurring *in vivo*. First, we generated glycine concentration-response curves for both wild type and mutant α3 GlyRs to confirm that our mutant contained high glycine sensitivity consistent with previous findings. Figure 2.4A shows the P185L mutation indeed resulted in GlyRs with significantly leftward shifted glycine concentration-response curves. However, the maximal glycine-activated currents were not significantly different. Next, we tested for differences in ethanol sensitivity between wild type and mutant α3 GlyRs. Figure 2.4B shows that the effects of 20 and 50 mM ethanol on wild type and mutant α3P185L GlyRs were not significantly different [for 20 mM ethanol: $t(3)= 0.5891$; $p> 0.05$; for 50 mM ethanol: $t(3)= 0.3352$; $p> 0.05$]. However, the effects of 200 mM ethanol were greater in wild type compared to mutant α3 GlyRs [$t(3)= 4.155$; $p< 0.05$].

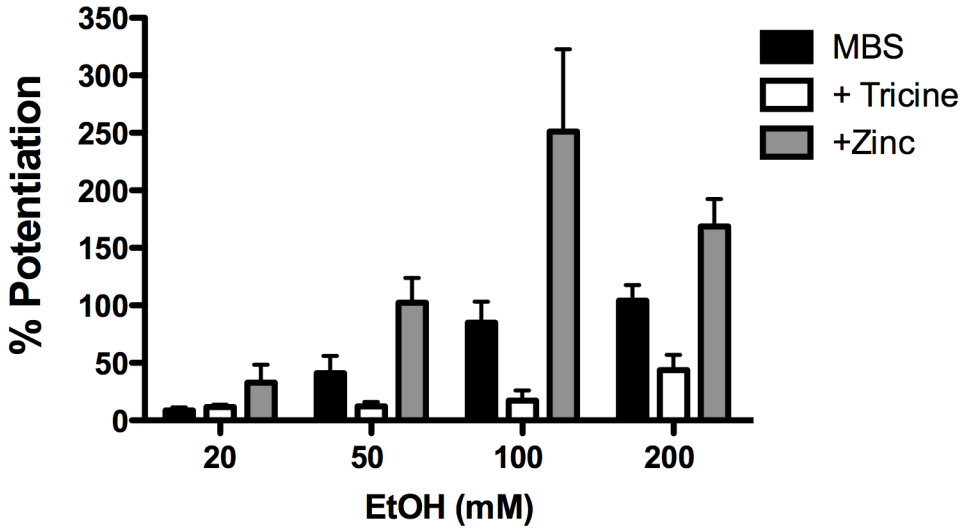

Figure 2.2: Ethanol modulation of α2 glycine receptors.
Ethanol modulation of submaximal glycine responses (EC5) was tested in α2 GlyRs. The effects of a series of ethanol concentrations (20, 50, 100, and 200 mM) were tested in standard MBS (containing contaminating levels of nanomolar zinc), in the presence of the zinc chelator tricine (no free zinc), and in MBS with 500 nM added zinc.

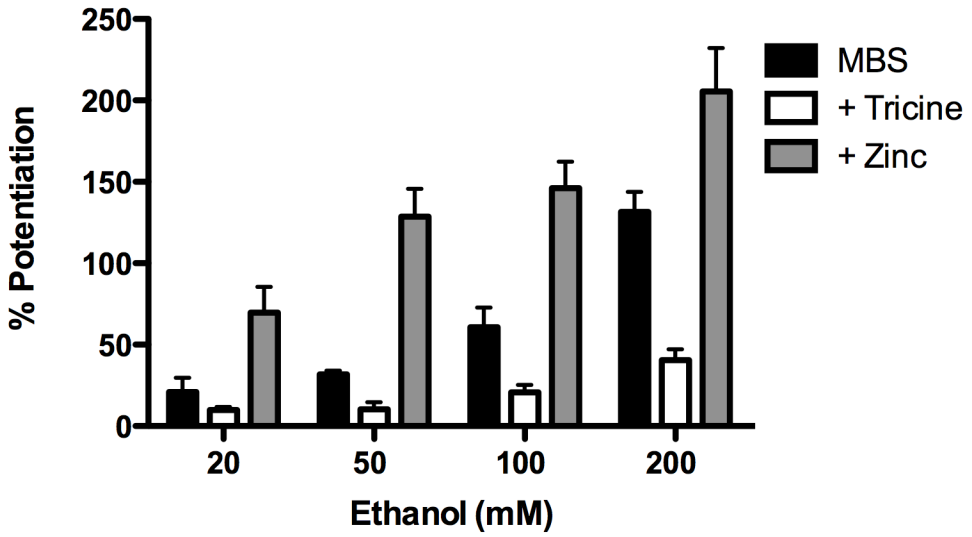

Figure 2.3: Ethanol modulation of α3 glycine receptors.
Ethanol sensitivity was confirmed in α3 GlyRs. The percent potentiation of submaximal (EC5) glycine responses by ethanol (20, 50, 100, 200 mM) was tested in standard MBS (containing nanomolar zinc contamination), in MBS plus the zinc chelator tricine (no free zinc), and in MBS containing 500 nM added zinc.

DISCUSSION

Zinc, which exists endogenously in the CNS at basal low nanomolar concentrations (Frederickson et al., 2006), allosterically modulates GlyR function such that nanomolar and low micromolar (< 10 μM) concentrations produce enhancement of glycine-activated currents, whereas higher micromolar concentrations of zinc (> 10 μM) produce GlyR inhibition (Bloomenthal et al., 1994; Harvey et al., 1999; Laube et al., 2000). In this study, manipulation of enhancing concentrations of zinc in our buffer solutions either by chelating zinc with tricine or by adding zinc resulted in changes in the effects of submaximal but not maximal concentrations of glycine on α2 and α3 GlyRs. This is consistent with previous findings in the α1 subunit (McCracken et al., 2010) and provides further evidence that low concentrations of zinc enhance GlyR function via allosteric modulation.

In the present study, we tested the hypothesis that the magnitude of ethanol's effect on α2 and α3 GlyRs is modulated by low physiological concentrations of zinc. We found that chelating contaminating zinc in our buffers, which we previously tested and reported to be less than 50 nM (McCracken et al., 2010), resulted in reduced effects of ethanol, whereas adding 500 nM zinc augmented the modulation of both α2 and α3 GlyRs by ethanol. This result is consistent with the enhancing effects of zinc on ethanol modulation of wild type α1 GlyRs that we previously reported (McCracken et al., 2010).

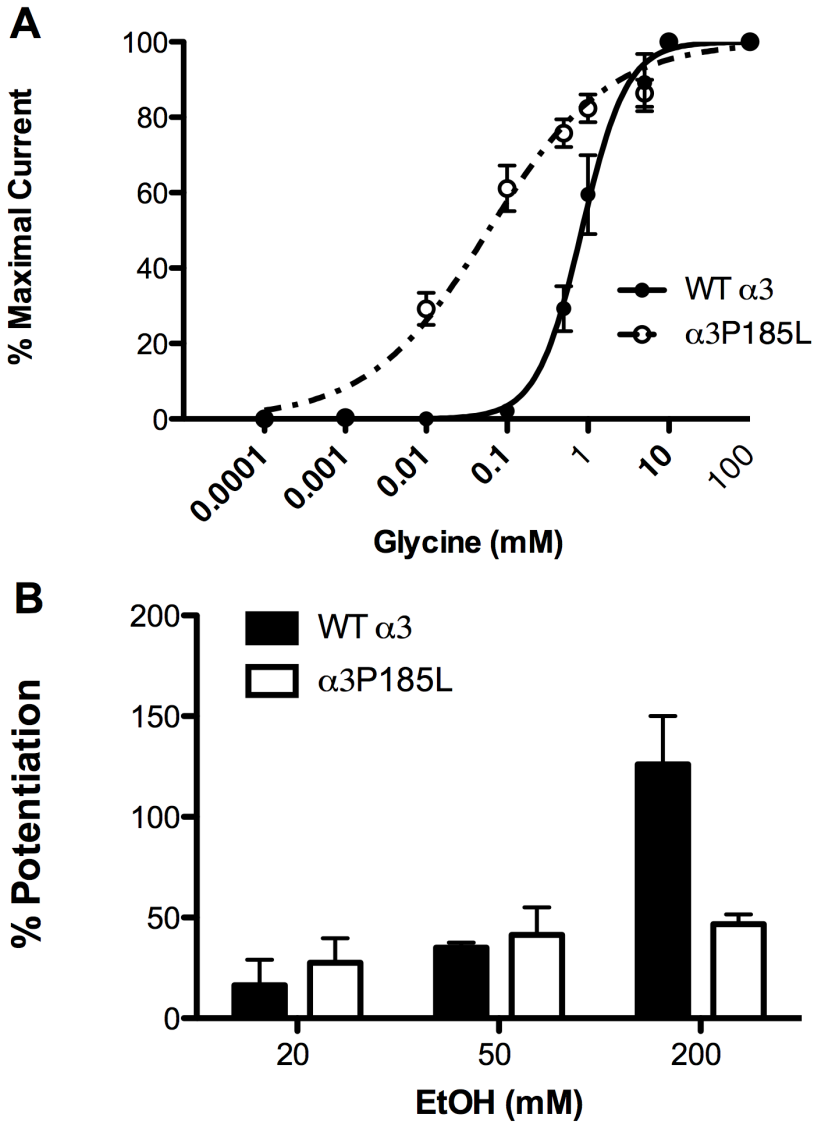

Figure 2.4: Glycine and ethanol sensitivity in α3P185L GlyRs.
A)Glycine concentration-response curves were generated for mutant α3P185L and wild type α3 GlyRs. Responses to a series of glycine concentrations (100 nM- 100 mM) were tested. B) The effects of ethanol (20, 50, and 200 mM) on submaximal glycine-activiated currents (EC5 glycine) were tested in wild type α3 and α3P185L GlyRs.

In addition, the overall importance of physiological zinc in determining the magnitude of ethanol's effect on GlyRs is further demonstrated in a recent study of a mutant α1 GlyR (M287L) with reduced sensitivity to ethanol. Although enhancement of α1M287L GlyRs by zinc alone is retained, zinc does enhance the effects of ethanol on the mutant receptor at nanomolar concentrations that suffice for increasing the magnitude of ethanol enhancement of receptor function in wild type GlyRs (Borghese et al., 2012).

The mechanisms for zinc modulation of GlyR function are not completely understood, but several amino acid residues located in the GlyR N-terminal domain seem to be key for the enhancing and inhibiting effects of zinc on GlyR function (Table 1). The potentiating effects of zinc, generally seen at concentrations in the nanomolar to low micromolar range (<10 μM), require high-affinity binding to amino acids distinct from those that have been identified as crucial for the inhibitory effects of concentrations of zinc in the higher micromolar range (> 10 μM) (Laube et al. 2000; Miller et al. 2005b). Notably, these higher (inhibitory) concentrations of zinc in the CNS are not tonically present, but may be achieved in instances of ischemia, seizure, trauma, and neurodegeneration (Choi & Koh,1998; Doraiswamy & Finefrock, 2004). For the purposes of this study, we focused on lower (nanomolar) enhancing concentrations of zinc that correspond more closely to zinc levels basally present in cerebrospinal fluid (Frederickson et al., 2006).

Alignment of the amino acid sequences for the GlyR α1, α2, and α3 subunits reveal that while most of the positions that have been demonstrated to be important for GlyR modulation by zinc are conserved across all three subunits,

there are positions that are not (see Table 1). For example, all of the putative zinc binding positions in the α1 GlyR are conserved at the homologous positions in the α2 and α3 subunits except for H107 in α1, which is asparagine-114 (N114) and asparagine-107 (N107) in α2 and α3 respectively. The significance of this difference can be seen such that homomeric α1 GlyRs are more sensitive to zinc inhibition than homomeric α2 or α3 GlyRs, but mutation of histidine-107 to asparagine results in an α1 H107N mutant that is less sensitive to zinc inhibition resembling α2 and α3 (Miller et al., 2005). In addition, D194 in α1 and α3, is not conserved at the homologous position in α2, which is glutamate-201 (E201). Studies of the enhancing effects of zinc on GlyRs have revealed the potential importance of this site in zinc binding and action. Homomeric α2 GlyRs are less sensitive to the potentiating effects of zinc than are homomeric α1 or α3 GlyRs. However, mutation of E-201 to aspartate (E201D) in α2 results in a mutant GlyR that is more sensitive to zinc enhancement, and thus resembles wild type α1 and α3 GlyRs (Miller et al., 2005). Despite the reported differences in zinc modulation of GlyR alpha subunits, it is important to note that nanomolar concentrations of zinc enhance ethanol's effects generalizes for all three alpha subunits (α1, α2, and α3) tested in this study and in our previous work.

Cysteine substitution experiments in the α1 GlyR at serine-267 (S267) in TM2 and alanine-288 (A288) in TM3, involving covalent thiol binding or cross-linking, suggest an alcohol and anesthetic binding pocket within the transmembrane domain of each subunit (Mascia et al., 2000; Lobo et al., 2006; 2008). Specifically, S267 and A288 were initially shown to be essential in GlyR enhancement by these agents (Mihic et al., 1997), and more recently it has been

shown that mutation of the neighboring positions Q266 and M287 also eliminates potentiation of GlyR function by ethanol (Borghese et al., 2012). Furthermore, findings from a series of studies indicate that amino acid residues in the TM1 and TM4 helices may also participate in alcohol and anesthetic binding (Lobo et al., 2004; 2006; 2008; McCracken et al., 2010).

Additional sites important for alcohol action on the α1 GlyR have also been suggested. These include alanine-52, which is in Loop 2 of the N-terminal domain (Mascia et al., 1996; Davies et al., 2004; Crawford et al., 2008), as well as lysine-385 (K385) of the large intracellular loop linking TM3 and TM4 (Yvenes et al., 2008).

Although less sensitive to the effects of alcohol than the α1 subunit, α2 GlyR function is also enhanced by ethanol, and of the GlyR alpha subunits it predominates in limbic brain regions affected by ethanol (McCool et al., 2003). The differences in alcohol sensitivity between the α1 and α2 subunits may reflect the presence of non-homologous residues in the N-terminal domain. For example, α1 GlyRs containing an alanine to serine substitution at position 52 (A52S) resemble wild type α2 receptors in their sensitivity to ethanol (i.e. the α1 A52S mutant is less sensitive than α1 wild type) (Mascia et al., 1996). In this study, we demonstrated that the α3 GlyR subunit does confer sensitivity to ethanol and that this effect is zinc-dependent. Although further investigations of alcohol modulation of α3 GlyRs are needed, it appears that modulation of the α3 GlyR subunit by zinc and ethanol is quite similar to the effects of these agents on the α1 GlyR subunit and may reflect the close homology of residues important for both the effects of ethanol and zinc.

An additional aim of this study was to test α3 GlyRs for differences in ethanol sensitivity between unedited and RNA edited receptors. We used site-directed mutagenesis to created an α3P185L GlyR that corresponds to RNA edited receptors occurring *in vivo*. Although we did not detect differences in the effects of low concentrations of ethanol (20 and 50 mM) on wild type and mutant α3P185L GlyRs, the edited receptors may be of interest for future investigations of the effects of lower concentrations of ethanol on Cys-loop receptors. Previous findings, as well as ours, show that the P185L receptors show higher affinity for glycine than unmodified or wild type α3 GlyRs (Meier et al., 2005; Legendre et al., 2009), and emergent evidence also suggests that RNA edited GlyRS are located non-synaptically (Eichler et al., 2009). In addition, sequence alignment with other members of the Cys-loop family reveal that the only other subunit that endogenously contains a leucine at the position homologous to 185 in GlyR α3 is the GABAA α6 subunit. However, there is functional homology at this position with the GABAA α6 and δ subunits, which also contain aliphatic residues, valine and alanine respectively, at this position. Like α3 GlyRs, these GABAA receptor subunits have high agonist sensitivities and are important for inhibitiory neurotransmission outside of the synapse (Belelli et al., 2009). Although controversial, there are reports that these GABAA receptor subunits contain increased sensitivities to low concentrations of ethanol relative to other GABAA receptors (Wallner et al., 2003; 2006; Borghese et al., 2005). Consequently, further studies of edited versus unedited α3 GlyRs may contribute valuable insights.

In conclusion, our findings demonstrate that like α1 and α2 GlyRs, α3 GlyRs are modulated by ethanol. In this study we show that the effects of ethanol on α2 and α3 GlyRs are zinc-dependent, as we have previously reported for α1 GlyRs. Furthermore, we provide evidence that it is critical to control for zinc levels in *in vitro* investigations of ethanol action at GlyRs. Finally, our findings suggest that accounting for the effects of zinc on ethanol action at GlyRs is important for understanding the sites and mechanisms of alcohol action as this may ultimately help contribute to improved treatments for alcohol use disorders. _

CHAPTER III: Mutation of a Zinc Binding Position (D80) on the α1 Glycine Receptor Subunit Changes Ethanol Sensitivity *In Vitro* and Alcohol Consumption *In Vivo*

INTRODUCTION

Strychnine-sensitive glycine receptors (GlyRs) are members of the Cys-loop superfamily of ligand-gated ion channels. Expressed in many brain regions including the cortex, hippocampus, amygdala, nucleus accumbens, striatum, ventral tegmental area (Jonsson et al., 2009; Baer et al., 2009), brain stem (Legendre, 2001), and cerebellum (Takahashi et al., 1992), as well as in the spinal cord (Legendre, 2001), GlyRs mediate inhibitory neurotransmission in the central nervous system (Legendre, 2001).

There are four known GlyR α subunits (α1, α2, α3, and α4) that are transcribed and translated from four distinct genes (*glra1, glra2, glra3 and glra4*) and one known β subunit (β from *glrb*) (Lynch et al., 2004), which can assemble to form pentameric homomers or heteromers containing an integral chloride channel. Structurally, each subunit contains a large extracellular N-terminus, an extracellular C-terminus, a large intracellular loop, and four transmembrane segments that collectively constitute a transmembrane domain.

Several agents act as allosteric modulators to enhance GlyR function including ethanol (and other alcohols), volatile anesthetics, and inhaled drugs of abuse (Mihic et al., 1997; Beckstead 2002). In addition to these exogenous modulators, endogenous agents like zinc, which is present in cerebrospinal fluid at tonic baseline concentrations in the low nanomolar range (Frederickson et al., 2006) also modulate GlyR function. However, it does so in a biphasic manner

such that nanomolar and low micromolar concentrations of zinc enhance GlyR function, whereas higher micromolar zinc concentrations produce inhibition (Bloomenthal et al., 1994; Laube et al., 1999; Miller et al., 2005).

In addition to modulating glycine-activated currents, recent evidence from *in vitro* investigations of recombinant GlyRs suggests that zinc also modulates ethanol action at GlyRs. More specifically, zinc chelation by tricine decreases the magnitude of ethanol enhancement of α1 GlyRs, whereas addition of physiologically relevant nanomolar concentrations of zinc enhances the magnitude of ethanol's effect at GlyRs (McCracken et al., 2010). However, the sites and mechanisms of action by which zinc and ethanol interact to modulate GlyR function are not presently understood and warrant further exploration.

Several lines of evidence highlight the physiological relevance of free zinc in the CNS and its ability to modulate GlyR function in the brain and spinal cord. Most notably are *Glra1*D80A knock-in (KI) mice, which contain a point mutation at a putative high affinity zinc binding site (D80) located in the N-terminal domain of the α1 GlyR subunit (Figure 3.1). Preliminary behavioral characterizations of mice homozygous for *Glra1*D80A reveal that they exhibit phenotypes analogous to human startle disease, and *in vitro* studies of spinal neurons and brainstem slices from these animals demonstrate significant impairments in the enhancement of spontaneous glycinergic currents by zinc (Hirzel et al., 2006). The availability of D80A mice affords the opportunity to study the potential role of zinc signaling at GlyRs in modulating alcohol drinking and other alcohol-related behaviors.

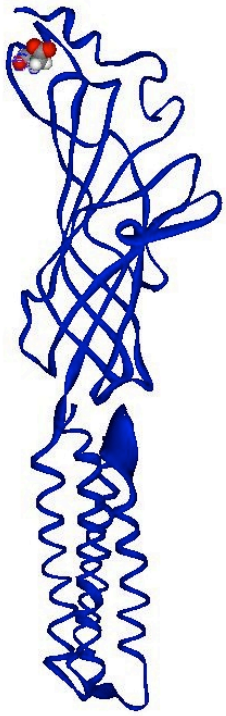

Figure 3.1: The D80 position of the α1 glycine receptor.
A schematic representation of an α1 GlyR subunit highlighting the aspartate residue at position 80 (D80; shown as red and gray spheres) in the N-terminal domain. In the absence of a GlyR X-ray crystal structure, homology modeling (based on the 2BG9 structure) was used to generate this illustration (Courtesy of Dr. James R. Trudell).

In the present study, we sought to investigate whether the high-affinity zinc binding position, D80, on the α1 GlyR subunit is important for the enhancing effects of zinc on ethanol modulation of GlyR function. We first conducted *in vitro* experiments using two-electrode voltage clamp electrophysiology to test the effects of ethanol, zinc, and other sedative agents on mutant α1D80A GlyRs expressed in *Xenopus* oocytes. Next, using the *Glra1*D80A KI mouse as an animal model, we evaluated the effects of a zinc-insensitive GlyR mutation on alcohol consumption and other related behavioral tests in mice. The combination of these *in vitro* and *in vivo* techniques were used to test the hypothesis that disruption of zinc signaling at GlyRs would produce changes in the effects of ethanol.

MATERIALS AND METHODS

Site-directed Mutagenesis

Site-directed mutagenesis was used to introduce a single point mutation in GlyR α1 cDNA to create the α1D80A mutant GlyR. This was accomplished using a QuikChange mutagenesis kit (Stratagene, La Jolla, CA) and commercially engineered mutagenesis primers (Integrated DNA Technology, San Diego, CA). Successful mutagenesis was verified using automated fluorescent DNA sequencing (The University of Texas at Austin DNA Core Facility, Austin, TX).

Xenopus Oocyte Isolation and cDNA Injection

Partial ovariectomies were performed on sexually mature female *Xenopus laevis* obtained from Xenopus Express (Brooksville, FL). Manual isolation of individual oocytes from ovary fragments, cDNA injection of isolated oocytes, and

incubation of injected oocytes were performed as previously described (McCracken et al., 2010).

Two-electrode Voltage Clamp Electrophysiology

For electrophysiology recordings, oocytes were impaled in the animal poles with two high-resistance (>1 MΩ) glass electrodes containing 3 M KCl and were voltage-clamped at -70mV using a Warner OC-725C oocyte clamp (Warner Instruments, Hamden, CT). A Masterflex USA peristalsis pump (Cole-Parmer Instrument Corporation, Vernon Hills, IL) was used to deliver buffer solutions to oocytes via bath perfusion at a rate of 2 ml/min. Clamping currents were recorded on LabChart Pro software (Colorado Springs, CO), which was interfaced to the oocyte voltage-clamp apparatus via a PowerLab 4/30 data acquisition system (AD Instruments, Colorado Springs, CO). Recordings were performed on oocytes expressing wild type α1 or mutant α1D80A mutant GlyRs approximately one to seven days post injection. All electrophysiology protocols were run using Modified Barth's Solution (MBS; 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$) in oocytes harvested from at least two different frogs.

Glycine Concentration-Response Curves. Glycine concentration-response curves were generated for oocytes expressing α1 and α1D80A GlyRs by measuring the chloride currents elicited by a series of glycine concentrations (10 μM-100 mM). Each concentration of glycine was applied for ~30 sec and was followed by a 7 min washout. The concentration of glycine that elicited the largest response was determined to be maximal, and the effects of the remaining

glycine concentrations were calculated and recorded as a percent of the maximal glycinergic effect. To confirm decreased sensitivity of α1D80A GlyRs to enhancing concentrations of zinc, additional glycine concentration-response curves were generated using the same protocol except that all MBS and glycine solutions contained 1 μM added zinc, which produces an approximately maximal enhancing effect of zinc on glycine-activated currents (Bloomenthal et al., 1994).

Taurine Concentration-Response Curves. Concentration-response curves for the GlyR partial agonist taurine were generated for wild type and mutant α1 GlyRs. A series of taurine concentrations (100 μM- 100 mM) was tested; each concentration was applied for ~30 sec and a 7 min washout period separated each application of taurine. The amount of current elicited by each taurine concentration was calculated and recorded as a percentage of the maximal glycine effect.

Zinc-dependent Modulation of Wild Type GlyRs by Ethanol. The effects of three different enhancing concentrations of zinc (100 nM, 500 nM and 1 μM) on the modulation of wild type receptors by ethanol were tested. Oocytes were first perfused with two ~15 sec applications of 10 mM glycine each followed by a 7 min washout period. The peak current elicited by the second application of glycine was considered to be the maximal response and was used to determine a concentration of glycine that produced ~5-10% (EC5-10) of the maximal glcyinergic effect. This experimentally derived EC5-10 concentration of glycine was then applied and following a 7 min washout period, 200 mM ethanol was first applied alone for 1 min, then it was applied concurrently with the experimentally derived EC5-10 concentration of glycine for ~45 sec. The EC5-10 concentration

of glycine was again applied alone for 45 sec following a 7 min washout. This procedure was carried out first in regular MBS, then it was repeated in MBS with 10 mM tricine to chelate contaminating free zinc, in MBS plus 100 nM added zinc, in MBS plus 500 nM added zinc, and in MBS plus 1 μM added zinc. In all conditions, the effects of ethanol were determined as percent potentiation of the glycine EC5-10 response.

Ethanol Sensitivity in Mutant vs. Wild Type GlyRs. Oocytes expressing either wild type α1 or mutant α1 D80A GlyRs were first perfused with two ~15 sec applications of 10 mM glycine each followed by a 7 min washout period. The peak current elicited by the second application of glycine was considered to be the maximal response and was used to determine a concentration of glycine that produced ~5-10% (EC5-10) of the maximal glycinergic effect. This experimentally derived EC5-10 concentration of glycine was then applied and following a 7 min washout period, ethanol was first applied alone for 1 min, then it was applied concurrently with the experimentally derived EC5-10 concentration of glycine for ~45 sec. The EC5-10 concentration of glycine was again applied alone for 45 sec following a 7 min washout. This protocol was used to test 50 mM and 200 mM ethanol.

To determine the effects of chelating contaminating zinc on ethanol modulation of wild type α1 and mutant α1D80A GlyRs lacking enhancement by physiological concentrations of zinc, we repeated the same protocol in the presence of 10 mM tricine, which was added to the buffer solution as previously described (McCracken et al., 2010). Because the level of free zinc is very low in this solution, we refer to it as "zinc free". In addition, to test the hypothesis that

added zinc would produce the opposite effect of a zinc chelator, this procedure was repeated for testing ethanol sensitivity in MBS containing 100 nM added zinc. In all three conditions, the effects of ethanol were determined as percent potentiation of the glycine EC5-10 response.

GlyR Modulation by Non-alcohol Sedatives. The effects of flurazepam, ketamine, pentobarbital, and 0.6 mM isoflurane in MBS were tested on wild type α1 and mutant α1D80A GlyRs. The same procedure that was used to test ethanol sensitivity was used to test wild type and mutant GlyR sensitivity to other non-alcohol sedatives.

Mouse Breeding and Genotyping

Breeding pairs of heterozygous *Glra1*D80A KI mice (backcrossed to C57BL/6 for at least five generations) were provided by Dr. H. Betz (Max-Planck Institute, Frankfurt, Germany). The mice used in this study were produced at The University of Texas from heterozygous breeding pairs. Following weaning, mice were housed at The University of Texas Animal Resource Center on a 12 hr light/dark cycle (lights on at 7:00 AM) with *ad libitum* access to standard rodent chow and water. All mice, male and female, used in behavioral tests were between eight and twelve weeks old and ethanol naïve at the start of each experiment and were used only once (i.e. animals were not used in more than one behavioral test). Due to poor viability and testing limitations resulting from the functional impairments of homozygous Glra1D80A KI mice, only heterozygous KI animals and their wild type littermate controls were included in the behavioral experiments. The protocols used for behavioral testing were approved by The

University of Texas Institutional Animal Care and Use Committee and were conducted in accordance with National Institutes of Health guidelines with regard to the use of animals in research.

Alcohol Consumption and Preference (24 hr access)

A standard two-bottle choice drinking protocol similar to those previously described (Blednov et al., 2003; 2012) was used. Mice were individually housed and given a one-week acclimation period. Two drinking tubes (one containing water and one containing an ethanol solution) were continuously available to mice. The tubes were weighed and their left/right positions were alternated daily (to avoid side preference). In addition, the mice were weighed every fourth day. Mice were offered 3% (v/v) ethanol versus water for two days. This was repeated successively for 6%, 9%, 12%, and 15 % (v/v) ethanol. The quantity of ethanol and water consumed by each mouse (g/kg body weight/ 24 hr) was calculated and the values for each respective concentration of ethanol were averaged. Throughout the experiment, estimates of spillage/evaporation were calculated daily from drinking tubes (one containing water and one containing the appropriate ethanol solution) placed in an empty cage on each row of the cage rack.

Non-alcohol Tastant Consumption and Preference (24 hr access)

In addition to ethanol, consumption of and preference for two other tastant solutions, saccharin and quinine, were tested in wild type and mutant KI mice to test for bitter or sweet taste preferences. In a two-bottle choice paradigm, mice were serially offered saccharin (0.0165% and 0.033%) and quinine hemisulfate

(0.03 mM and 0.066 mM) and their intakes were calculated daily. Each respective concentration was offered for two consecutive days, and the bottle positions were alternated daily. For each tastant, the lower concentration was always presented first followed by the higher concentration, and one week separated tastant testing during which mice had access to two bottles, both containing water.

Drug Preparation and Injection

All drug solutions were prepared as previously described (Blednov et al., 2011). Ethanol (Aaper Alcohol and Chemical, Shelbyville, KY or Pharmco, Brookfield, CT) solutions were prepared in 0.9% saline (20% v/v) and injected i.p. at a dosing volume of 0.2 ml/10g of body weight. Flurazepam (Sigma-Aldrich, St. Louis, MO) and pentobarbital (Sigma/RBI, Natick, MA) were dissolved in 0.9% saline and were injected i.p. at 0.01ml/g of body weight. Strychnine (Sigma-Aldrich, St. Louis, MO) was prepared in 0.9% saline and was injected i.p. at a volume of 10ml/kg of body weight.

Loss of Righting Reflex (LORR)

Wild type and KI mice were tested for sensitivity to the sedative effects of ethanol (3.4 and 3.8 g/kg) and other CNS depressants including flurazepam (225mg/kg), pentobarbital (50 mg/kg), and ketamine (175 mg/kg) using a standard duration of LORR (sleep time) assay as previously described for other GlyR KI mice (Blednov et al., 2012). Once ataxic, mice were placed in the supine position in V-shaped plastic troughs until they were capable of righting themselves three time within 30 sec; sleep time was defined as the time from

when they were place in the supine position until they regained their righting reflex.

Rotarod

Mice were trained on a fixed speed (5.0 rpm) rotarod (Economex; Columbus Instruments, Columbus, OH). Training was considered complete when mice could remain on the rotarod for 60 sec. To test the effects of ethanol on motor coordination, wild type and KI mice were administered ethanol (2.0 g/kg i.p.) and 15 min post injection they were placed back on the rotarod. Their latency to fall was recorded until they were capable of remaining on the rotarod for 60 sec.

Acoustic Startle Response

SR-LAB test stations and software (San Diego Instruments, San Diego, CA) were used to test acoustic startle responses in wild type and mutant KI mice. Startle responses elicited by a series of auditory stimuli were record as previously described (Findlay et al., 2003; Blednov et al., 2012). First, mice were placed in Plexiglas holding chamber for a 5 min acclimation period. Then, in the following 8 min, mice were presented with seven trial types across five discrete blocks of trials for a total of 30 trials. There was a 10-20 sec inter-trial interval. To control for baseline movement, one trial type measured the response to no stimulus, whereas the remaining six trial types measured the response to startle stimuli. This consisted of 40 msec sound bursts of 90, 95,100, 105, 110, or 115 dB. Starting at the onset of startle stimuli, startle amplitude was measured every 1 msec during a 65 msec period. The six trial types were presented to mice in a

pseudorandom order such that each trial type was presented once within a block of six trials. The maximum startle amplitude (Vmax) over this sampling period was taken as the dependent variable. A background noise level of 70 dB was maintained throughout the duration of the test session.

Strychnine-induced Convulsions

Wild type and KI mice were injected with a series of doses of the GlyR antagonist strychnine to test for differences in chemically-induced seizure activity. For each dose of strychnine, a group of five mice was used. During testing, mice were individually placed into observation chambers (20 cm X 10 cm X 10 cm Plexiglas cage), and were continuously observed following injections of strychnine (up to 30 min post injection) for latency to clonic and tonic convulsions. The data were recorded and tabulated as previously described (Blednov et al., 2012) as the number of mice exhibiting the behavioral signs as well as the latency to first seizure occurrence. The dose of strychnine that elicited convulsive activity in 50% of the group (ED50) was calculated using linear regression analyses.

Statistical Analysis

For experiments in recombinant GlyRs, nonlinear regression analyses were performed to calculate glycine EC50 values and Hill coefficients for glycine concentration-response curves, and t-tests and one-way ANOVAs followed by post-hoc analyses were used to determined differences in ethanol sensitivity and the effects of other sedative agents. In addition, two-way ANOVAs (with Dunnett's or Bonferroni post hoc tests), Student's t-tests, and linear regression

analyses were conducted to detect differences between groups in the behavioral experiments. Overall, statistical differences were determined at $p< 0.05$, and all analyses were conducted using GraphPad PRISM software (San Diego, CA).

RESULTS

Recombinant GlyRs Expressed in Oocytes

Glycine Sensitivity. We first generated agonist (glycine and taurine) concentration-response curves for α1 and α1D80A GlyRs expressed in oocytes to determine differences in agonist sensitivity. For the full agonist glycine, introducing the D80A mutation in the α1 subunit resulted in a rightward shift in the glycine concentration-response curve for the mutant compared to wild type GlyRs (Figure 3.2A). To confirm the results of previous studies that show that mutant α1D80A GlyRs have reduced sensitivity to enhancement of glycine-activated currents by low micromolar and nanomolar concentrations of zinc, we also generated glycine concentration-response curves in the presence of 1 μM added zinc. This concentration of zinc, which is approximately maximally enhancing on wild type GlyRs (Bloomenthal et al., 1994), did not produce any shifts in the glycine concentration-response curve of mutant α1D80A GlyRs indicating no enhancement of the glycine-activated currents. In addition, there was no significant difference in the maximal glycine-activated currents in mutant versus wild type receptors in either normal MBS or in the presence of 1 μM added zinc.

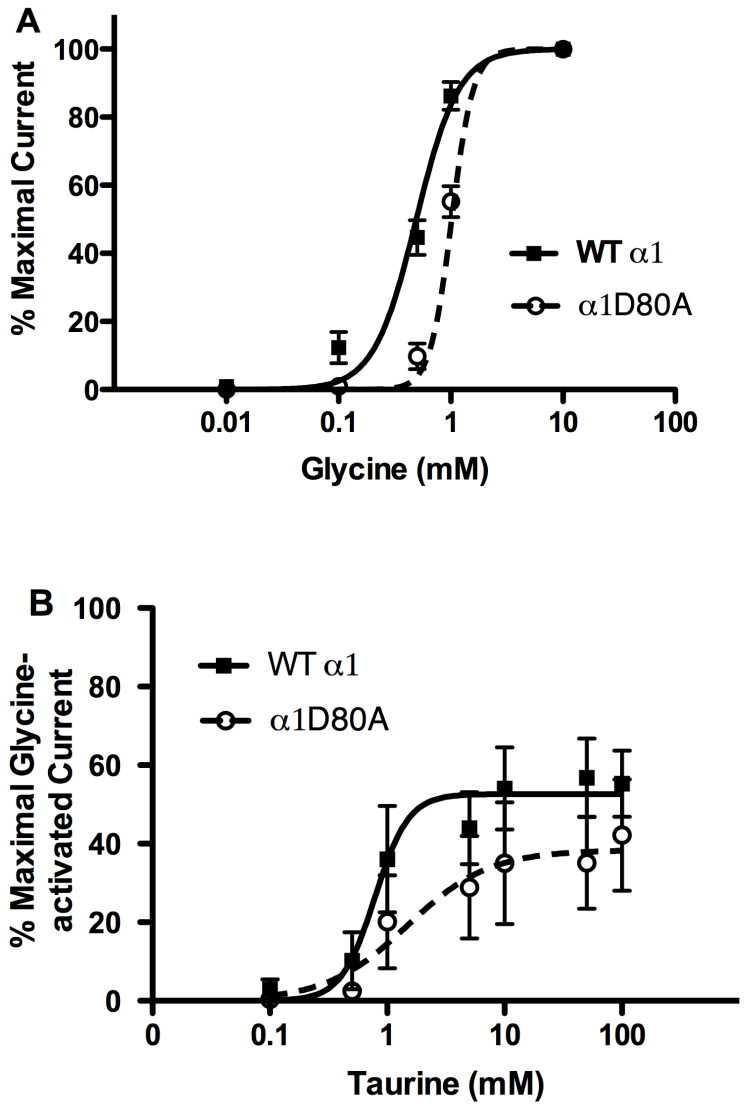

Figure 3.2: α1D80A glycine receptor sensitivity to agonists.
Agonist concentration response curves for mutant α1D80A and wild type α1 GlyRs. A) A series of glycine concentrations (10μM- 10 mM) were tested on mutant and wild type GlyRs. B) A series of taurine concentrations (100 μM- 100 mM) were also tested. no significant difference in the maximal glycine-activated currents in mutant versus wild type receptors in either normal MBS or in the presence of 1 μM added zinc.

Taurine Sensitivity. Concentration-response curves for the partial agonist taurine suggested that it might be less efficacious on mutant α1D80A GlyRs compared to wild type GlyRs, however, this trend was not statistically significant (Figure 3.2B).

Zinc Modulation of Ethanol Sensitivity in Wild Type GlyRs. Emergent evidence shows the importance of zinc in determine the effects of ethanol on GlyRs and because the D80 position is among the known high affinity sites important for enhancement of GlyR function by low physiological concentrations of zinc, we tested the hypothesis that mutation of this site would produce changes in the effects of ethanol on mutant α1D80A GlyRs expressed in oocytes. To do this, we first tested the effects of 200 mM ethanol on wild type α1 GlyRs with a series of different concentrations of zinc ranging from a zinc-free MBS solution that contained the zinc chelating agent tricine to 1 μM zinc to determine which concentration produced the greatest increase in ethanol enhancement. Manipulating the levels of zinc in our perfusion solutions significantly changed the magnitude of ethanol's enhancing effect on wild type α1 GlyR function (Figure 3.3A). In zinc-free MBS, which contained 10 mM tricine, 200 mM ethanol had the smallest degree of enhancement of glycine-activated currents. In contrast, ethanol modulation of GlyR function was greatest in the presence of 100 nM added zinc. Intermediate degrees of enhancement were observed when the effects of 200 mM ethanol were tested in the presence of 500 nM and 1 μM added zinc.

Ethanol Sensitivity of Mutant D80A GlyRs. Next, we investigated whether mutation of the aspartate at position 80 to alanine in the α1 GlyR would result in

GlyRs with decreased sensitivity to ethanol. The enhancement of wild type α1 and mutant α1D80A GlyRs by 50 mM and 200 mM ethanol in standard MBS solutions was measured. Figures 3.3B and 3.3C show that the mutant compared to wild type GlyRs demonstrated decreased sensitivity to 50 mM [$t(7)= 2.422$; $p= 0.0459$] and 200 mM [$t(14)= 2.358$; $p= 0.0334$] ethanol, respectively.

Zinc/Ethanol Interactions in Wild Type and D80A GlyRs. To determine if the decreased ethanol sensitivity of the D80A mutant might be due to the loss of the enhancing effects of zinc on ethanol action, we subsequently tested the effects of the same ethanol concentrations (50 mM and 200 mM) in mutant and wild type receptors first in the presence of the chelating agent tricine and then in the presence of 100 nM added zinc, which in the previous experiment we determined to produce the greatest increase in the magnitude of ethanol's effect on wild type α1 GlyRs. Manipulating the concentrations of zinc present in our buffers did not change in the magnitude of ethanol enhancement of mutant α1D80A GlyRs by either 50 mM or 200 mM ethanol [for 50 mM ethanol: $F(2,9)= 1.147$; $p= 0.3601$; for 200 mM ethanol: $F(2,12)= 0.6007$; $p= 0.5641$] (Figure 3.3B and C). In contrast, for wild type GlyRs, the presence of tricine significantly decreased the degree of enhancement by 50 mM and 200 mM ethanol, and the opposite effect was observed such that 100 nM added zinc significantly increased the effects of both 50 mM and 200 mM ethanol on wild type GlyR function [for 50 mM ethanol: $F(2,7)= 191.5$; $p< 0.0001$; for 200 mM ethanol: $F(2,17)= 8.941$; $p= 0.0022$].

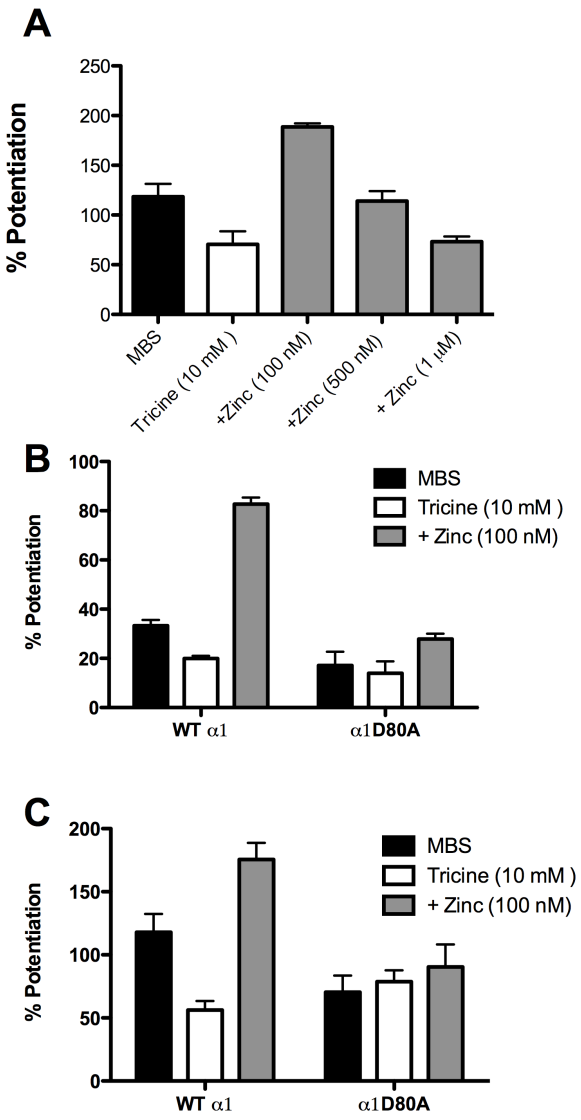

Figure 3.3: α1D80A glycine receptor sensitivity to ethanol.
Modulation of mutant D80A and wild type α1 GlyRs by ethanol in the presence and absence of zinc. A) The effects of 200 mM ethanol on α1 GlyRs were tested in standard MBS, in tricine, and in three added zinc conditions (100 nM, 500 nM, and 1 μM). B) Modulation of D80A and wild type GlyRs by 50 mM ethanol was tested in standard MBS, in tricine, and in 100 nM added zinc. Similarly, the modulation of D80A and wild type GlyRs by 200 mM ethanol was measured in three conditions: MBS, MBS with tricine, and MBS with added zinc.

Sensitivity of Mutant D80A GlyRs to Other Sedative Agents. In addition to ethanol, we tested the effects of other sedative agents on wild type α1 and α1D80A GlyRs. There were no differences in the enhancement of wild type or mutant GlyRs by flurazepam, pentobarbital, ketamine, or isoflurane [$t(7)= 0.5293$; $p= 0.6129$].

Behavioral tests in GlyR KI Mice

Mouse Breeding. Heterozygous breeding pairs of *Glra1*D80A KI mice were used to produce the animals tested in this study. Mice homozygous for the D80A mutation display low viability, and we used heterozygous *Glra1*D80A mice and their respective wild type litters mates all the behavioral tests conducted. More specifically, of 138 mice that we produced 46 mice or 33% were wild type (+/+), 71 mice or 52% were heterozygous (+/-) for the D80A mutation, and 21 mice or 15% were *Glra1*D80A homozygotes (-/-). Chi-square analysis of the number of mice we produced with each respective genotype reveals that the actual genotype ratios that we generated were significantly different than those predicted by Mendelian genetics [$\chi^2(2, N= 138)= 9.174$; $p= 0.0102$]. The small number of homozygotes that we were able to produce, were impaired in a manner that would have occluded reliable measurements in most of the behavioral tests that we conducted.

Alcohol Consumption and Preference. A continuous access, two-bottle choice drinking paradigm was used to test for differences in alcohol consumption and preference between D80A GlyR KI mice and wild type littermate controls. Female *Glra1*D80A mice consumed significantly less ethanol than wild type

females [main effect: $F(4, 64)= 2.90$; $p= 0.0286$; $t(17)= 3.064$; $p< 0.05$ at 9% ethanol] and also exhibited decreased preference for alcohol [$F(4,64)= 9.52$; $p< 0.0001$]. However, this effect was sex-specific as there were no differences in ethanol consumption [$F(4,85)= 1.06$; $p= 0.3813$] or preference [$F(4,85)= 0.70$; $p= 0.5913$] between male wild type and mutant mice. There were also no significant differences in total fluid intake detected between the two genotypes for males or females [for males: $F(4,85)= 0.24$; $p= 0.9146$; for females: $F(4,64)= 0.30$; $p= 0.8754$ (Figure 3.4).

Non-alcohol Tastant Consumption and Preference. To test for differences between mutant and wild type mice with respect to sweet or bitter tastants, we used a continuous access two-bottle choice paradigm to measure saccharin and quinine consumption and preference. There were no significant differences detected between D80A KI and wild type male or female mice for either saccharin consumption [for females: $F(1,16)= 0.68$; $p= 0.4212$; for males: $F(1,16)= 0.04$; $p= 0.8532$], preference [for females: $F(1,16)= 0.84$; $p= 0.3718$; for males: $F(1,16)= 0.0$; $p= 0.9757$], or total fluid intake [for females: $F(1,16)= 0.47$; $p= 0.5018$; for males: $F(1,16)= 0.13$; $p= 0.7252$] (Figure 3.5). In addition, Figures 3.6A and B show that female D80A KI and wild type mice did not differ in their consumption [$F(1,16)= 0.0$; $p= 0.9662$] or preference [$F(1, 16)= 0.0$; $p= 0.9944$] for quinine. However, in this assay the mutant D80A mice did show less total fluid intake compared to wild type females [$F(1, 16)= 7.48$; $p= 0.0147$] (Figure 3.6C).

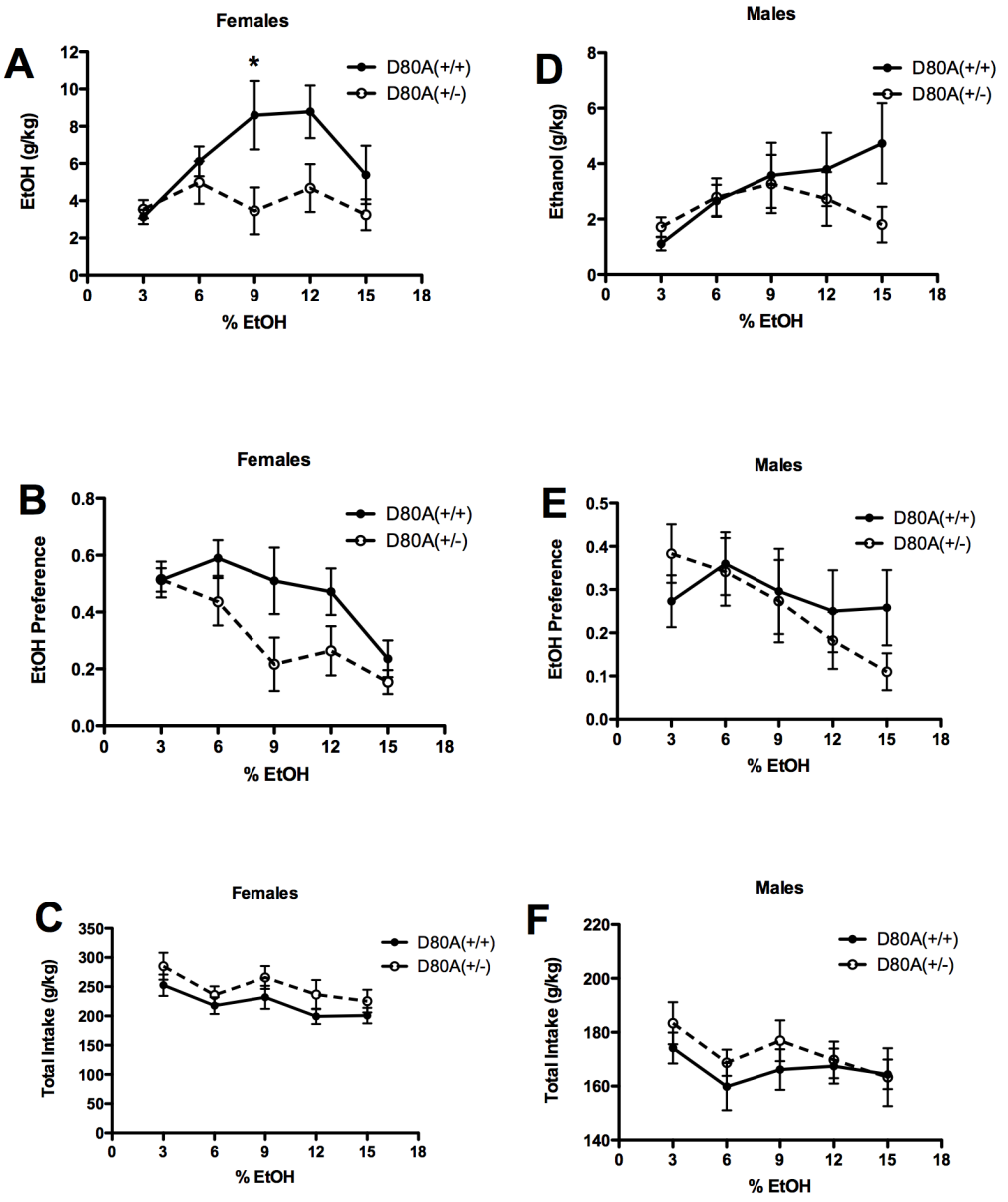

Figure 3.4: Ethanol consumption and preference in mutant and wild type mice. Ethanol consumption (g/kg/24hrs) and preference were measured in heterozygous D80A KI mice and their wild type littermates. A and B) Both consumption and preference were decreased in female D80A mice. However, this effect was sex-dependent. D and E) There were no differences in ethanol consumption or preference for wild type and D80A mutant mice. C and F) Total fluid intake was not different between wild type and D80A mutant mice.

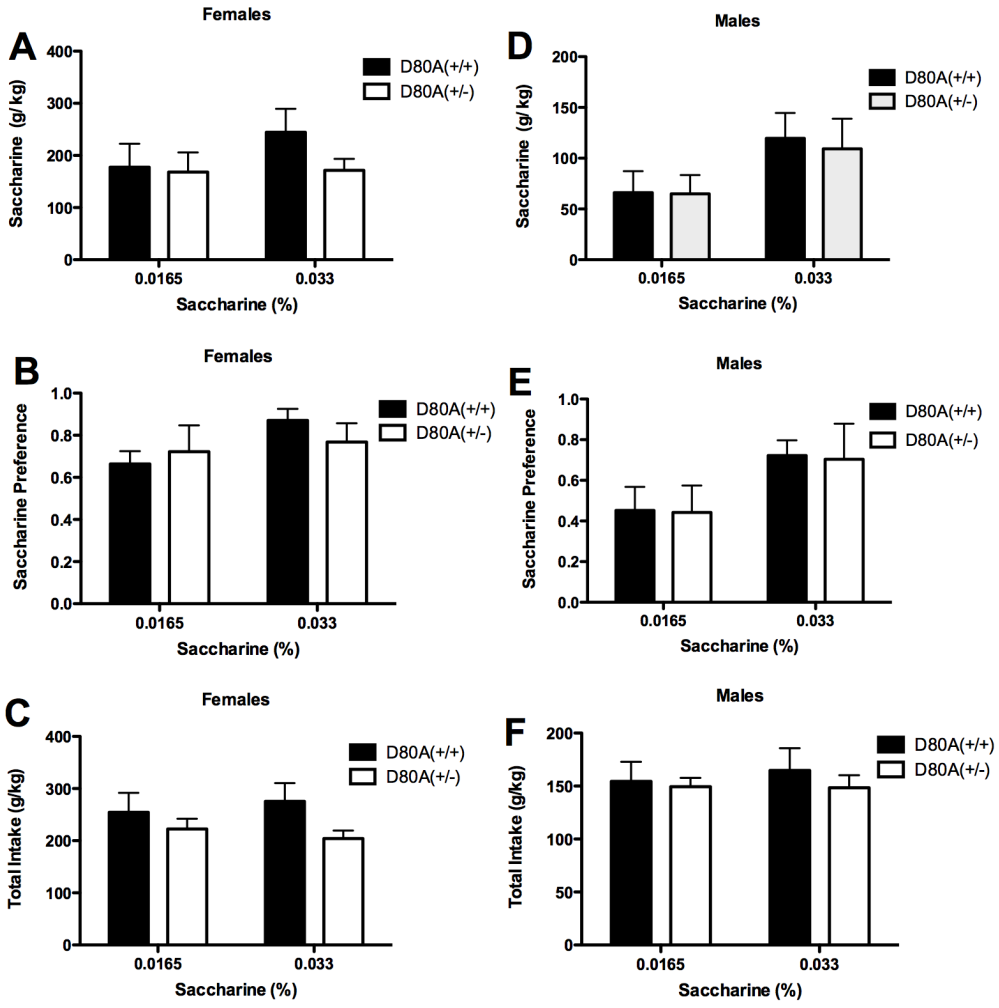

Figure 3.5: Saccharin consumption and preference in mutant and wild type mice. Consumption (g/kg/24hrs) of and preference for the sweet tastant saccharin were tested in D80A and wild type mice. Neither females nor males displayed differences in consumption (A and D). Similarly, no differences in preference were detected (B and E). C and F) There were no differences in total fluid intake in this assay.

Male mutant and wild type mice did not demonstrate any differences in either quinine consumption [F(1,16)= 0.24; p= 0.6344], preference [F(1, 16)= 0.59; p= 0.4554], or total fluid intake [F(1,16)= 0.13; p= 0.7252] (Figure 3.6E, F, and G).

Loss of Righting Reflex. Differences between mutant and wild type mice in the duration of LORR was measured following the injection of four sedative agents, ethanol, flurazepam, pentobarbital, or ketamine. For ethanol (Figures 3.7A and B), there was no effect of genotype on sleep time for either males [t(16)= 0.1491; p= 0.8834] or females [t(16= 1.979; p= 0.0652] (Figure 3.7A and B). However, male D80A KI demonstrated increased LORR compared to their wild type male littermates in response to pentobarbital [t(8)= 4.008; p= 0.0039], flurazepam [t(9)= 10.56, p< 0.0001], and ketamine [t(10)= 7.211; p< 0.0001] (Figures 3.7C, E, and G). Likewise, Figures 3.7D, F and H show that the same effect was observed in female mice in response to pentobarbital [t(12)= 7.510; p< 00.001], flurazepam [t(15)= 3.990; p= 0.0012], and ketamine [t(12)= 11.40; p< 0.0001], respectively.

Acoustic Startle Response. Behavioral characterizations of other GlyR KI mice show that impairments in GlyR function are accompanied by changes in startle responses (Findlay et al., 2003; Blednov et al., 2012). Therefore, we compared this behavior in D80A KI and wild type mice. In both sexes, the mutant mice exhibited increased startle responses compared to wild type controls. There were not main effects for males [F(4, 110) = 0.67; p= 0.6113] or females [F(4,60)= 2.14; p= 0.0861].

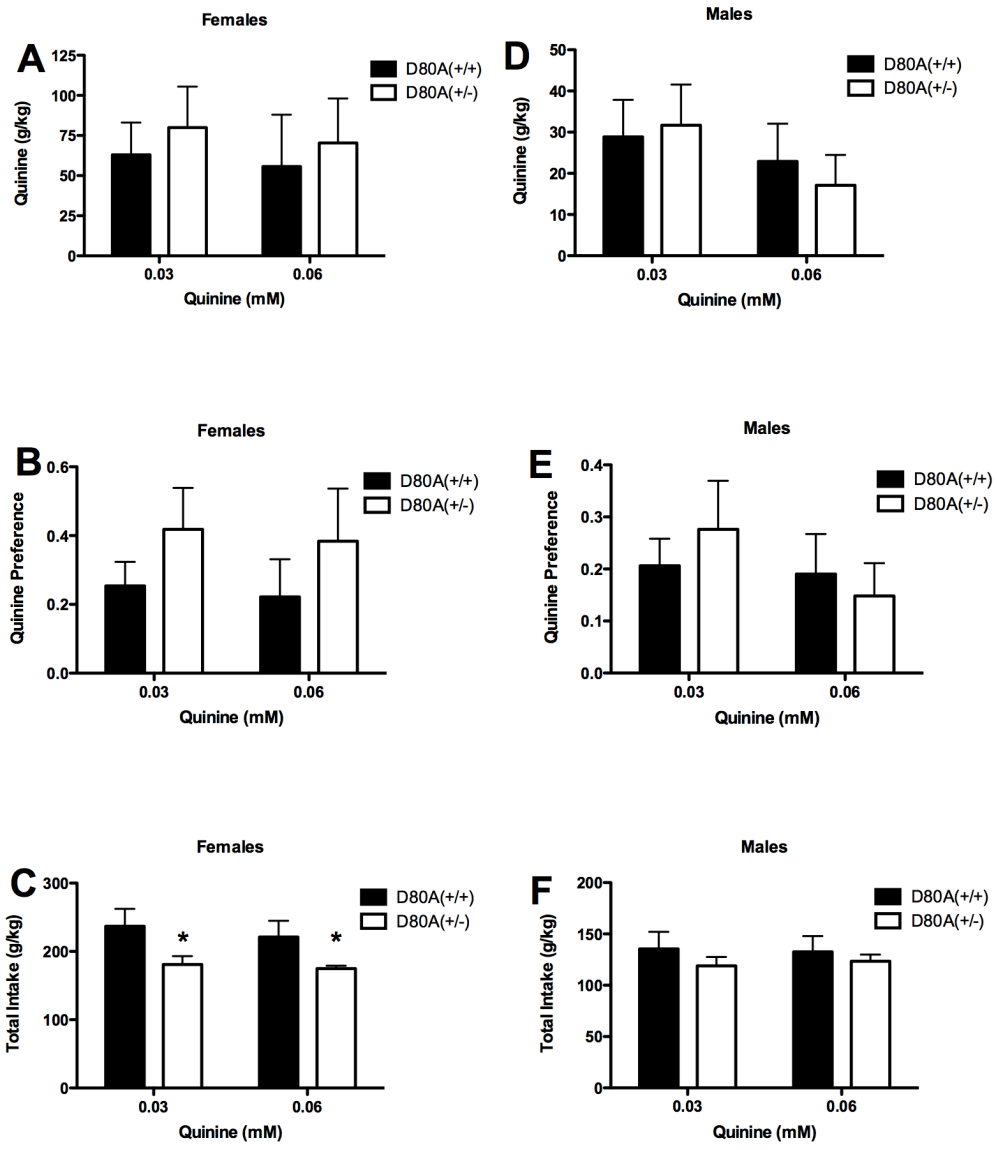

Figure 3.6: Quinine consumption and preference in mutant and wild type mice. Consumption (g/kg/24hrs) of and preference for the bitter tastant quinine were tested in D80A and wild type mice. Neither females nor males displayed differences in consumption (A and D). Similarly, no differences in preference were detected (B and E). C) Female D80A had decreased total fluid intake compared to wild type mice. However, there were no differences in total fluid intake for males in this assay.

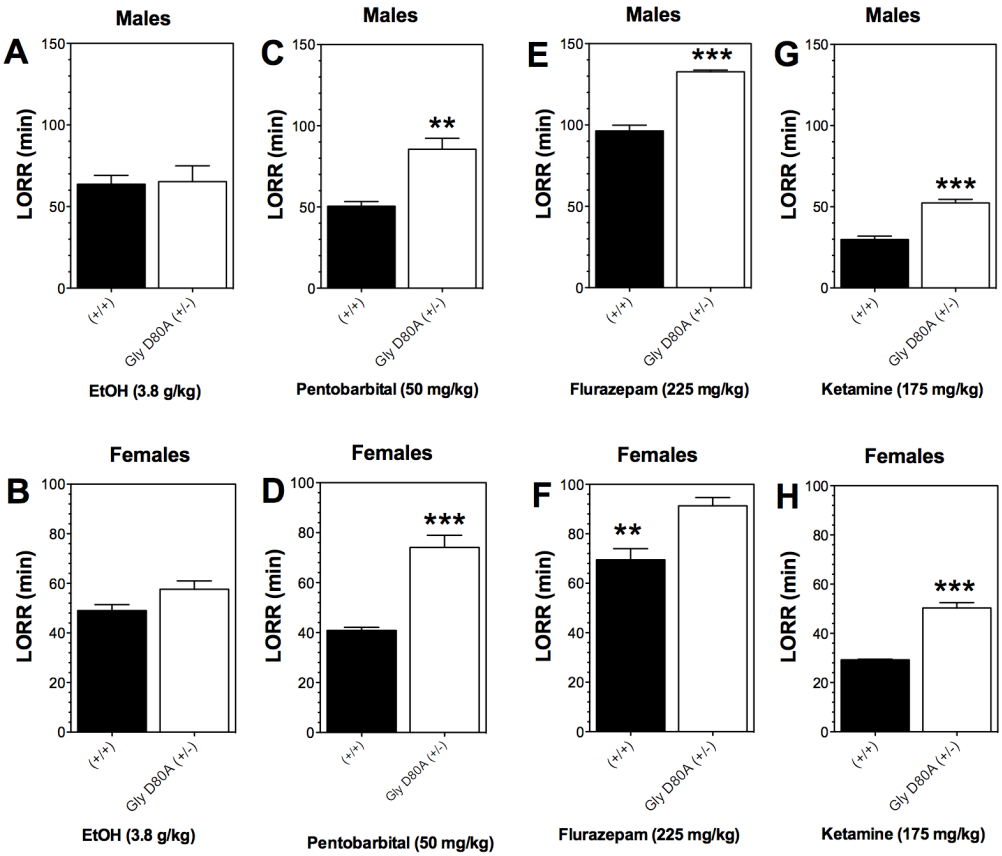

Figure 3.7: Loss of righting reflex induced by sedative agents.
The duration of loss of righting reflex (LORR) induced by ethanol, pentobarbital, flurazepam, and ketamine were measured in D80A KI and wild type mice. A and B) There were no differences in ethanol sleep time between mutant and wild type mice. However, both male and female D80A mice had increased durations of LORR induced by pentobarbital (C and D), flurazepam (E and F), and ketamine (G and H).

However, there were differences detected at individual decibel levels for males [$t(24)= 2.833$; $p< 0.05$; at 115 dB] and females [$t(13)= 3.530$; $p< 0.01$ at 110 dB; $t(13)= 4.366$; $p< 0.01$ at 115 dB] (Figures 3.8A and B). In an additional series of experiments, we further tested whether ethanol modulation of acoustic startle responses was changed in mutant D80A KI mice and discovered that ethanol (0.5 or 1.0 g/kg, i.p.) had no effect on startle responses of female [$F(10, 222)= 0.10$; $p= 0.9998$] and male [$F(10, 156)= 0.67$; $p= 0.7488$] mutant KI mice(Figure 3.8C and E). However, both ethanol doses increased startle responses in male WT mice [$t(25)= 2.739$; $p< 0.05$ for 0.5 g/kg ethanol; $t(25)= 2.812$; $p< 0.05$ for 1.0 g/kg ethanol] (Figure 3.8D), whereas only 0.5 g/kg ethanol increased startle responses in female WT mice [$t(21)= 3.643$; $p< 0.01$] (Figure 3.8F).

Ethanol Rotarod. To measure the motor-incoordinating effects of ethanol (2 g/kg, i.p.), we used the rotarod test . This dose of ethanol impaired motor coordination, however, there were no measureable differences between female [$F(7, 80)= 0.63$; $p= 0.7271$] or male [$F(9, 100)= 0.23$, $p= 0.9889$] mutant and wild type mice in their respective recoveries from ethanol-induced incoordination (not shown).

Strychnine Convulsions. We measured tonic convulsions in wild type and mutant D80A KI mice induced by injections of the GlyR antagonist strychnine. There were no differences observed between wild type and KI mice in sensitivity (ED50) to tonic convulsions in either females (0.49 mg/kg for wild type vs. 0.54 mg/kg for KI mice) or males (0.53 mg/kg for wild type vs. 0.59 for KI mice).

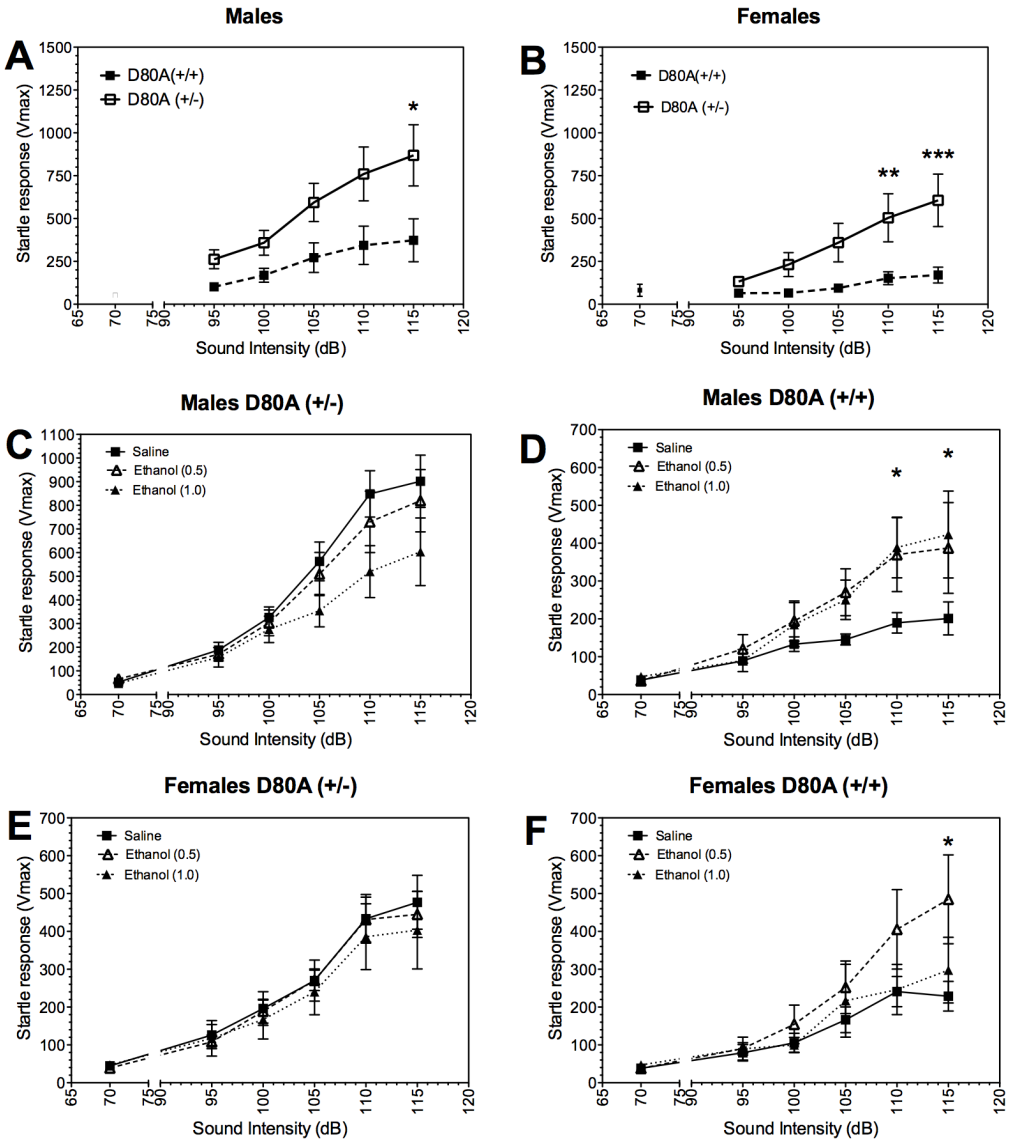

Figure 3.8: Acoustic startle responses in mutant and wild type mice.
The data represent the maximum startle amplitude (Vmax) as a function of sound intensity in decibels (dB). A and B) The startle responses female and male D80A mice were increased compared to wild type controls. The effects of ethanol on startle responses were also measured C and D) Ethanol increased the startle responses in male wild type, but not mutant D80A mice. Likewise, female wild type mice had increased startle responses following injections of ethanol, but this was not observed in female D80A mice (E and F).

DISCUSSION

Although the mechanism for zinc modulation of GlyR function is not completely understood, several amino acid residues located in the *N*-terminal domain of the α1 subunit responsible for the enhancing and inhibiting effects of zinc on GlyR function have been identified. The potentiating effects of zinc, generally seen at concentrations in the nanomolar to low micromolar range (<10 μM), require high-affinity binding to amino acids at positions aspartate-80 (D80), threonine-151 (T151), glutamate-192 (E192), aspartate-194 (D194), and histidine-215 (H215) (Laube et al. 2000; Miller et al. 2005b).

In the present study, we first characterized *in vitro* the effects of introducing a mutation (D80A) at one of these high-affinity zinc binding sites. The decrease in affinity for sub-maximal glycine that we observed in the α1D80A mutant versus wild type GlyRs in this study is consistent with the effects of the zinc chelating agent tricine on decreasing glycine affinity in wild type receptors. McCracken et al. (2010) used tricine to eliminate low nanomolar concentrations of contaminating zinc and showed that this resulted in wild type GlyRs with right shifted glycine concentration-response curves of similar magnitude to the shifts observed for the α1D80A GlyRs tested in our current study. In addition, the lack of difference observed between the taurine concentration response curves of wild type and mutant GlyRs is consistent with previous reports that the α1D80A mutation reduces enhancement of glycine-activated, but not taurine-activated currents by nanomolar and low micromolar concentrations of zinc (Laube et al., 2000; Miller et al., 2005). Furthermore, neither manipulating zinc concentrations nor introducing the D80A mutation produced changes in maximal glycine-

activated currents, which serves as further evidence that zinc modulates GlyR function as an allosteric modulator.

Although zinc concentrations in the brain exceed those present in other organs, most brain zinc is protein-bound (Mathie et al. 2006). In its free or rapidly exchangeable form, zinc exists in cerebospinal fluid at tonic baseline concentrations ranging from approximately 5-25 nM (Frederickson et al. 2006). However, in the CNS, additional zinc is secreted from neurons (Takeda et al., 2001), and this can result in transient zinc concentrations in excess of 1 μM following presynaptic release from GABAergic, glutamatergic, or glycinergic terminals (Frederickson et al. 2001).

Because physiological concentrations of zinc enhance the magnitude of ethanol's effects on α1 GlyRs (McCracken et al., 2010), we tested whether mutant α1D80A GlyRs would confer decreased sensitivity to ethanol. The enhancing effects of both 50 and 200 mM ethanol were reduced in mutant D80A GlyRs compared to wild type. Just as the glycine concentration-response curves for α1D80A GlyRs resembled previously published wild type glycine concentration response-curves in the presence of the zinc chelator tricine, the reduced effects of ethanol enhancement on D80A mutant GlyRs also seemed to mimic the effects of ethanol observed in wild type α1 GlyRs in the presence of zinc chelation by tricine.

To determine whether the reduced sensitivity of α1D80A GlyRs to ethanol was due to the elimination of the enhancing effects of zinc on ethanol action, we ran additional experiments in which the effects of ethanol were tested again in the presence of a zinc chelator (tricine) and in the presence of added zinc

(100 nM). The results from these experiments showed that unlike wild type α1 GlyRs in which chelating zinc decreases the effects of ethanol and adding physiological nanomolar zinc increases ethanol enhancement, manipulating the concentrations of zinc in which ethanol was tested on mutant α1D80A GlyRs did not change the magnitude of ethanol's effect. This suggests that the D80 position on the α1 GlyR subunit is critical for zinc modulation of ethanol action. In addition, recent pharmacological characterization of another GlyR α1 subunit mutant (M287L), also with decreased ethanol sensitivity, revealed that zinc did not enhance the effects of ethanol on mutant receptors at nanomolar concentrations that were suffice for increasing the magnitude of ethanol enhancement of receptor function in wild type GlyRs (Borghese et al., 2012). This further indicates that zinc is crucial in determining the sensitivity of GlyRs to ethanol and highlights the importance of including zinc in studies of ethanol receptor pharmacology.

Several studies in rodent models of ethanol consumption provide compelling evidence indicating a role for GlyRs in alcohol drinking and ethanol reinforcement. For example, bilateral infusions of glycine into the nucleus accumbens increase dopamine release and reduce ethanol drinking and preference, whereas infusions of the GlyR antagonist strychnine decrease dopamine output and increase ethanol intake (Molander et al., 2005). Similarly, injections of two different glycine reuptake inhibitors, Org 25935 and Org 24598, each produce reductions in ethanol intake, and Org 25935 also reduces ethanol preference (Molander et al., 2007; Lido et al., 2011).

A logical extension of these findings is that increased brain zinc might increase ethanol consumption. Indeed, genetic studies of alcohol drinking in mice show a negative correlation between zinc levels in brain and ethanol consumption (Jones et al., 2008).

The apparent importance of both GlyRs and zinc in alcohol consumption paired with our *in vitro* data indicating a critical role for zinc in modulating ethanol action at GlyRs provided a rationale to study the potential role of zinc signaling at GlyRs in alcohol consumption and other alcohol-related behaviors. In addition, *Glra1*D80A KI mice, which contain the same zinc mutation that we characterized *in vitro*, provided us with an animal model for studies of zinc/ethanol interactions at the behavioral level. From these experiments, a few distinct differences were observed between mutant KI and wild type mice. First, D80A KI mice had decreased consumption of and preference for ethanol compared to their littermate controls. However, this effect was only observed in females as the males showed no difference, but is consistent with the sex-differences also observed in reduced alcohol consumption and preference for other GlyR KI mice previously studied (Table 2) (Findlay et al., 2003; Blednov et al., 2012). Furthermore, our finding is also consistent with the genetic studies discussed above that revealed a correlation between levels of hippocampal zinc and alcohol consumption in female, but not in male mice (Jones et al., 2008).

Additional behavioral differences between D80A KI and wild type mice include LORR induced by pentobarbital, flurazepam, and ketamine. Both female and male mutant mice had increased sleep times following injections of the above drugs. This effect also is consistent with the results of behavioral tests in other

GlyR mutant mice (Table 2) (Findlay et al., 2003; Blednov et al., 2012), suggesting that impairments in normal GlyR function potentially lead to compensatory changes in the receptors of other neurotransmitter systems such as GABA or glutamate. Although we did not quantitate changes in GlyR expression levels in wild type versus D80A KI mice in this study, findings from the initial characterizations of mice homozygous for the mutation did not show differences in strychnine binding assays performed on tissue from mutant D80A and wild type mice suggesting that mice with the D80A mutation do not contain increased or decreased numbers of GlyRs (Hirzel et al., 2006).

Multiple GlyR mutations in mammals, both naturally occurring and engineered, result in enhanced startle phenotypes (Findlay et al., 2003; 2005; Harvey et al., 2008; Blednov et al., 2012). Like these other GlyR mutants, D80A KI mice also showed increased acoustic startle responses compared to wild type mice, which is consistent with results from the preliminary non-alcohol related behavioral characterizations of these mice (Hirzel et al., 2006). In addition, we tested the effects of ethanol on startle responses and found that it had no effect on the startle responses of mutant KI mice, but did increase the startle responses of WT mice indicating that zinc signaling and the D80 position on the α1 GlyR maybe important in mediating ethanols effects on startle responses.

Homozygous KI mice carrying each of the previously characterized GlyR KI mutations (S267Q, Q266I, and M287L) were not viable, and therefore all ethanol-related behavioral tests of these mutations have been limited to heterozygous animals (Findlay et al., 2002; 2003; 2005; Blednov et al., 2012).

		Gly α1 (M287L)		Gly α1 (Q266I)		Gly α1 (Osc)		Gly α1 (S287Q)		Gly α1 (D80A)	
Test	Behavior	M	F	M	F	M	F	M	F	M	F
Startle reflex		↑	↑	↑↑	↑↑	↑=	↑=	↑↑	↑↑	↑	↑
Startle reflex	EtOH activation	↓	↓	↓	↓					↓	↓
LORR (ethanol)	3.8 g/kg	↓	=	↑	↑	↑	=	=	↑	=	=
LORR (pentobarbital)	50 mg/kg	↑	↑	=	=					↑↑	↑↑
LORR (ketamine)	175 mg/kg	↑	↑	↑	↑		=			↑	↑
LORR (flurazepam)	225 mg/kg	↑	↑	↑↑	↑↑		=			↑	↑
	EtOH Intake	=	↓	=	↓	=	=	↑	=	=	↓
2 Bottle choice	Prefer.	↓	↓	↓	↓	=	↓	=	=	=	↓
(ethanol)	Fluid Intake	↑	↑	↑	↑	=	↑	↑	↑	=	=
2 Bottle choice	Prefer.	=	=	↓	=	=	=	↓	=	=	=
(saccharin)	Fluid Intake	=	=	↓	=	=	=	↑	↑	=	=
2 Bottle choice	Prefer.	↓	↓	=	=	=	=	=	↓	=	=
(quinine)	Fluid Intake	=	=	↑	↑	=	↓	↑	↑	=	↓
Rotarod (recovery)	EtOH (2 g/kg)	=	=	←	←			←	←	=	=
Convulsions	Strychnine	↑		↑						=	=

Table 2: Comparison of behavioral phenotypes for heterozygous GlyR KI mice. Increases in behavior are indicated by ↑, decreases are represented with ↓, leftward shifts in time-dependent recovery curves are expressed as ←, and = denotes no difference between the respective mutant and their wild type littermate controls. Data for M287L and Q2661 are from Blednov et al. (2102), Osc data are from Kling et al. (1997) and Findlay et al. (2003), and S267Q data are from Findlay et al. (2003). There are conflicting findings for Osc mice as Kling et al. (1997) reported increases in startle, whereas Findlay et al. (2003) found no differences. For blank spaces, there are no published reports characterizing the given behavioral phenotype for that particular genotype.

Likewise, we tested wild type and heterozygous *Glra1*D80A mice because our colony yielded a low number of homozygotes and the motor impairments of those that we did produce would have occluded measurements in the behavioral tests that we ultimately performed. The overall level of impairment observed in homozygous D80A KI mice highlights the importance of GlyR modulation by endogenous zinc in normal neurological function.

In this study, we focused on one of amino acid resides of the α1 GlyR subunit with known importance for the enhancing actions of zinc on GlyR function. However, additional residues of the GlyR α1 subunit, in particular histidine-107 (H107), histidine-109 (H109), threonine-112 (T112), and threonine-133 (T133) are thought to contribute to lower-affinity binding sites and are necessary for inhibition of GlyR function by higher micromolar concentrations of zinc (> 10 μM) (Harvey et al., 1999; Laube et al., 2000; Miller et al., 2005). The importance of zinc binding at lower affinity (GlyR inhibitory) sites is illustrated in instances of ischemia, seizure, trauma, and neurodegeneration, during which zinc levels are estimated to peak in the brain at concentrations in excess of 100 μM (Choi & Koh,1998; Doraiswamy & Finefrock, 2004). Future studies focusing on these higher concentrations of zinc and the corresponding binding positions on the GlyR may be important for additional characterizations of zinc in modulating ethanol action at the GlyR.

Overall, our findings demonstrate the significance of low physiological concentrations of zinc in modulating the effects of ethanol on GlyR function and more specifically the crucial role of the D80 position in the α1 GlyR subunit in regulating this effect both *in vitro* and *in vivo*. In addition, the results from our

behavioral tests in mutant D80A GlyR KI mice provide additional evidence that impairments in GlyR function correspond to changes in alcohol-related behaviors. Finally, these data elucidate the role of zinc in determining alcohol action at GlyRs, and by better understanding the sites and mechanisms of alcohol action, we can ultimately develop more efficacious treatments for alcoholism and other alcohol-related health complications.

CHAPTER IV: Discussion, Implications, And Future Directions

Despite zinc existing more abundantly in brain than any other organ of the body, previous investigations of the effects of ethanol on ion channel function have not examined the potential role of this endogenous agent in ethanol receptor pharmacology. The overall goal of this project was to address this issue by specifically investigating the effects of zinc on alcohol action at the GlyR using *in vitro* and *in vivo* techniques. A zinc/ethanol interaction was first characterized in recombinant GlyRs, and then, a site of action underlying zinc modulation of ethanol action at GlyRs was examined using recombinant receptors containing a mutation at a known zinc-binding site (D80A). In addition, behavioral studies of the effects of alcohol in mice containing zinc-insensitive mutant GlyRs were tested.

The initial aim of this work was to test whether zinc is important for ethanol modulation of GlyR function. Our preliminary work revealed that physiological (nanomolar) concentrations of zinc that correspond to the levels of zinc tonically present in cerebrospinal fluid act to enhance the magnitude of ethanol's effect at α1 GlyR subunits (McCracken et al., 2010). In this series of studies, we sought to extend these findings to other GlyR alpha subunits, α2 and α3, that are expressed throughout the brain and spinal cord (Baer et al., 2009; Jonsson et al., 2009). As described in greater detail in chapter 2, our data demonstrate that nanomolar concentrations of zinc also act to enhance the effects of ethanol on α2 and α3 GlyRs. For example, removing all zinc with the chelator tricine decreased ethanol modulation of these GlyRs, whereas adding nanomolar

concentrations of zinc increased the effects of ethanol as we previously observed in α1 GlyRs. Prior to this work, there were no published reports of ethanol modulation of α3 GlyRs, however, in this study we showed that like the more thoroughly studied α1 and α2 GlyR subunits, α3 GlyRs are also allosterically modulated by intoxicating concentrations of ethanol and that the magnitude of ethanol's effect on these channels is zinc-dependent.

The findings from this work in combination with evidence from studies of GlyR gene and receptor membrane expression provide compelling evidence of the necessity for future investigations of the mechanisms and sites of action of ethanol to include or even primarily focus on α2 and α3 GlyRs. For example, quantification of GlyR subunit mRNA levels (Table 1) demonstrates that of the GlyR alpha subunits present in brain, the α2 subunit predominates in the mammalian forebrain, which includes limbic and reward centers such as the nucleus accumbens and amygdala (Jonsson et al., 2009). For example, Delaney et al. (2010) show that in the amygdala the levels of α2 subunit protein expression at cellular membranes is the highest of the GlyR alpha subunits expressed in this region suggesting that there is a greater abundance of actual receptors not just higher RNA message levels.

Furthermore, studies that used glycine receptor pharmacology to manipulate ethanol consumption and reinforcement provide additional evidence that although a majority of past studies in the alcohol field have largely focused on the α1 GlyR subunit future work should place greater emphasis on α2 and α3 GlyRs.

GlyR		Amyg	Ant Hypo	Cing Gyrus	Caud Puta	Nuc Acc	PFC	Post Hypo	VTA
α1	AA	0.15±0.05	0.31±0.08	0.04±0.02	0.03±0.01	0.03±0.01	-	0.58±0.14	1.41±0.34
	ANA	0.14±0.05	0.37±0.10	0.06±0.02	0.02±0.01	0.04±0.02	-	0.71±0.23	1.11±0.52
α2	AA	0.11±0.02	0.14±0.05	0.11±0.03	0.36±0.15	0.46±0.12	0.15±0.03	1.16±0.22	0.71±0.15
	ANA	0.09±0.02	0.13±0.04	0.08±0.02	0.48±0.21	0.51±0.12	0.12±0.03	1.26±0.31	0.62±0.11
α3	AA	0.26±0.08	0.24±0.05	0.06±0.03	0.04±0.03	0.31±0.09	0.05±0.02	1.26±0.43	0.31±0.08
	ANA	0.25±0.06	0.22±0.07	0.04±0.02	0.04±0.02	0.26±0.09	0.06±0.03	1.19±0.23	0.59±0.29
β	AA	0.85±0.19	0.16±0.03	0.37±0.10	1.09±0.20	0.75±0.08	0.55±0.11	0.94±0.24	2.28±0.79
	ANA	0.70±0.14	0.17±0.05	0.38±0.10	0.95±0.29	0.87±0.16	0.47±0.09	0.94±0.20	2.63±1.35

Table 3: Glycine receptor expression levels in brain.
The mean mRNA expression levels (± standard deviations) for GlyR α1, α2, α3, and β subunits in the amygdala (Amyg), the anterior hypothalamus (Ant Hypo), the cingulate gyrus (Cing Gyrus), the caudate putamen (Caud Puta), the nucleus accumbens (Nuc Acc), the prefrontal cortex (PFC), the posterior hypothalamus (Post Hypo), and the ventral tegmental area (VTA) of Alko Alcohol (high drinking) and Non-Alcohol (low drinking) rats. The expression levels represent values normalized to the mean of three reference genes (β-actin, GAPDH, and RPL19) (adapted from Jonsson et al., 2009).

Briefly, Soderpalm and colleagues conducted a series of experiments that show that increasing glycine levels in the nucleus accumbens (a brain region in which α2 and α3 GlyR subunits predominate relative to α1 GlyRs) of rodents either directly by bilateral injects of actual glycine or indirectly by injections of glycine uptake inhibitors (Org25935 and Org24598) results in increased levels of dopamine and decreased ethanol consumption. Conversely, injections of the GlyR antagonist strychnine into the accumbens reduced dopamine output and increased alcohol drinking (Molander et al., 2005; 2007; Lido et al., 2011) suggesting a role for GlyRs in mediating the reinforcing effects of ethanol. In a broader context, it has been proposed that GlyRs expressed in the mesolimbic dopamine system may be in the future a novel access point for studies of drug reinforcement (Soderpalm et al., 2009). However, at this time much more experimentation is needed.

Despite the predominance of α2 GlyRs, and to a lesser extent α3 GlyRs, in forebrain regions involved in alcohol-related behaviors, all GlyR KI mice that have been engineered to investigate the behavioral effects of mutant GlyRs with decreased ethanol sensitivity contain point mutations in the GlyR α1 subunit gene, *Glra1*. With the exception of the D80A KI mice used as an animal model in this study, all others have contained single amino acid substitutions in the GlyR α1 subunit transmembrane domain (specifically in TM2 or TM3). In addition, study of alcohol drinking and other related behaviors in animals homozygous for each of the respective mutations were prevented by impaired viability (Findlay et al., 2002; 2003; 2005; Blednov et al., 2012). For D80A KI mice, initial behavioral tests included mice homozygous for the mutation (Hirzel et al., 2006),

however, in this study, we were unable to study homozygous D80A mice due to our colony yielding a low number of homozygotes. In addition, their seizure phenotype and impaired movement capabilities occluded reliable measurements in most behavioral tests.

In contrast to the viability limitations and impaired phenotypes of mice with genetic alterations in the α1 GlyR subunit, both α2 and α3 knock-out mice are viable and overtly normal (Young-Pearse et al., 2006; Harvey et al., 2004). This combined with the expression levels of α2 and α3 GlyR subunits in forebrain regions involved in alcohol-related behaviors suggests that mice with alterations the GlyR α2 or α3 subunit genes may be more appropriate and valuable as animal models for future studies of GlyR function in mediating alcohol action and reinforcement. In addition, a logical extension of the findings from studies of GlyR mutations in the α1 subunit with reduced ethanol sensitivity could be introducing corresponding mutations at the homologous positions in α2 and α3 GlyRs. These could then serve as substrates of interest for the future construction of GlyR KI mice. Furthermore, an alternative approach to constructing and testing GlyR KI or KO mice would be to use RNAi to produce brain-region specific knock-down of a receptor subunit of interest. This would circumvent the time, cost, and viability issues that have plagued studies of GlyR function in mediating alcohol-related behaviors in the context of constructing and testing GlyR KI mice.

In addition to reports of GlyR agonists and antagonists, such as glycine and strychnine, modulating alcohol consumption in rodents, there is also recent evidence that acamprosate, a widely prescribed medication for the maintenance of

abstinence in alcohol dependence, may also reduce alcohol drinking via a GlyR-mediated mechanism in the nucleus accumbens (Chau et al., 2010a; 2010b; Lido et al., 2011). However, in initial studies of recombinant α1 GlyRs, there were no effects of acamprosate (Riley et al., 2008). Subsequent studies of relative gene and receptor membrane expression levels of the GlyR α subunits in the mammalian forebrain revealed that the inconsistency between the published *in vitro* and *in vivo* findings may be a reflection of the predominant expression of α2 and α3 GlyRs relative to α1 GlyRs in the nucleus accumbens. Accordingly, we recently tested the hypothesis that α2 or α3 GlyRs, because they are the more abundant GlyR alpha subunits in the nucleus accumbens (Jonsson et al., 2009), would confer sensitivity to acamprosate in the absence of an effect on the α1 subunit. Figure 4.1 shows that like the α1 GlyR subunit, neither the α2 nor the α3 GlyR subunit is significantly modulated by acamprosate. This suggests that the *in vivo* effects of acamprosate may be due to an indirect mechanism involving GlyRs, glycine, or glycine transporters rather than a direct effect on GlyRs expressed in the nucleus accumbens. Alternatively, the effects observed in the nucleus accumbens could also be a downstream or indirect effect of action in another brain region with either efferent or afferent projections with the nucleus accumbens. Regardless, this highlights the importance of developing improved GlyR-related pharmacotherapies with known mechanisms of action.

Differences in expression levels are not the only distinguishing features among GlyR alpha subunits. Several recent studies provide evidence of the functional differences among these proteins. A profound example is the α3 GlyR subunit, which undergoes post-transcriptional modifications (RNA editing and

splice variations) and is involved in inflammatory pain via modulation by prostaglandins (Meier et al., 2005; Eichler et al., 2009; Harvey et al., 2004). These respective features provide α3 GlyRs with distinguishing functional implications. For example, α3 transcripts are RNA-edited to produce α3P185L GlyRs with significantly higher affinity for glycine than unedited α3 GlyRs (Meier et al., 2005) and localization and functional studies suggest that they are largely expressed extra-synaptically where they are involved in tonic inhibition (Eichler et al., 2008; 2009). Sequence alignments of the GlyR subunits with other members of the Cys-loop superfamily reveal that the only other subunit that endogenously contains a leucine at the position homologous to 185 in GlyR α3 is the GABAA α6 subunit. However, there is functional homology at this position with the GABAA α6 and δ subunits, which also contain aliphatic residues, valine and alanine respectively, at this position. Like α3 GlyRs, these particular GABAA receptor subunits have high agonist sensitivities and are important for inhibitory neurotransmission outside of the synapse (Belelli et al., 2009).

Although controversial, there are reports that these GABAA receptor subunits contain increased sensitivities to low concentrations of ethanol relative to other GABAA receptors (Wallner et al., 2003; 2006; Borghese et al., 2005). Due to the functional distinctions between α3 GlyRs and the other GlyR alpha subunits and their similarities to particular GABAA receptors, we tested for preliminary differences in ethanol sensitivity between wild type α3 and mutant α3P185L GlyRs expressed in oocytes. There were no differences in ethanol sensitivity at low to moderate concentrations (20 and 50 mM) of ethanol between wild type and mutant α3 receptors.

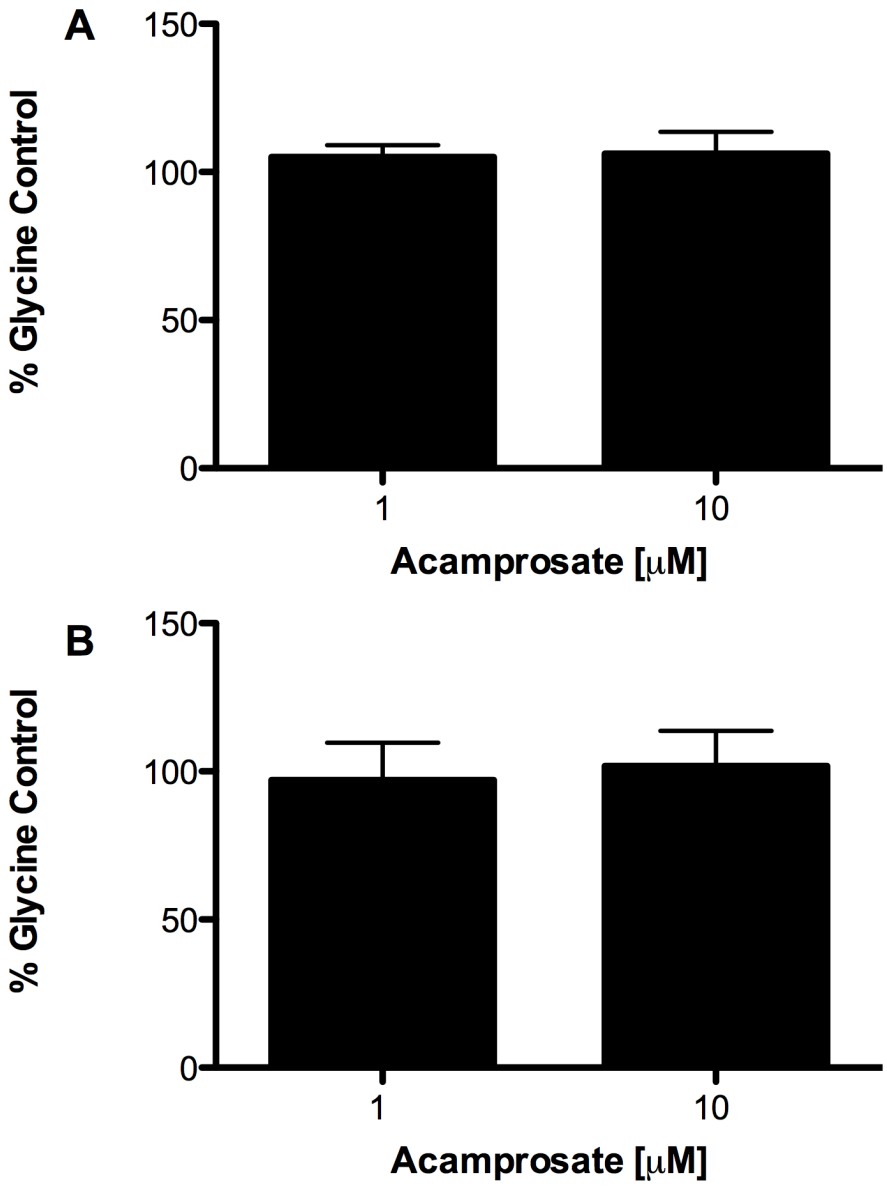

Figure 4.1. Acamprosate modulation of α2 and α3 GlyRs.
The effects of 1 and 10 μM acamprosate were tested on A) α2 and B) α3 GlyRs expressed in *Xenopus* oocytes. Acamprosate was pre-applied for 1 min and then was concurrently applied with an EC5 concentration of glycine for 45 sec. The data represent percent potentiation of the glycine-activated (EC5) currents.

This is in contrast to the reports that suggest α4, α6, and δ containing GABAA receptors confer high sensitivity to low concentrations of ethanol (Wallner et al., 2003; 2006; but see also Borghese et al., for conflicting results). In addition, it suggests that edited α3 GlyRs may be only functionally similar to a subset of GABAA receptors in terms of agonist sensitivity and extra synaptic localization, but that amino acid residues of the N-terminal domain Loop 2 region are in fact critical for determining high sensitivity to low ethanol concentrations. This is further supported by findings from a GlyR α1 receptor chimera containing the Loop 2 region of GABAA δ receptors with sensitivity to low millimolar concentrations of ethanol (Perkins et al., 2009). Consequently, further pharmacological and functional studies of edited versus unedited α3 GlyRs may contribute valuable insights into the sites and mechanisms of ethanol action due to their similarities and differences to both GABAA and other glycine receptors.

The second aim of this study was to explore a possible site of action important for zinc modulation of ethanol action at the GlyR. Although we do not have a detailed understanding of the mechanism by which zinc biphasically modulates GlyR function, several amino acid residues in the extracellular N-terminal domain of the α1 GlyR subunit are critical for both the enhancing and inhibiting effects of zinc on glycine-activated currents. As described in detail in earlier chapters, these include positions D80, T151, E192, D194, and H215 for zinc enhancement and positions H107, H109, T112, and T133 for zinc inhibition (Laube et al., 2000; Miller et al., 2005b). The D80 position was our initial candidate position for investigation as it was the original amino acid residue that was shown to reduce zinc enhancement of glycine-activated currents when

mutated (Laube et al., 2000). In addition, the availability of D80A KI mice (Hirzel et al., 2006) provided the opportunity to ultimately expand any findings from *in vitro* experiments to include behavioral studies of the potential importance of zinc signaling at GlyRs in alcohol consumption and related behaviors.

Mutation of the D80 position to alanine resulted in GlyRs with altered responses to glycine and ethanol that closely resembled findings that we published previously demonstrating the effects of chelating contaminating zinc on glycine and ethanol responses in wild type α1 GlyRs. Both the addition of tricine to our buffer solutions and the introduction of the D80A mutation resulted in rightward shifts in the glycine concentration-response curves and reduced enhancement of GlyR function by ethanol. However, in mutant α1D80A GlyRs the addition of nanomolar concentrations of zinc did not enhance the magnitude of ethanol's effect as it did at wild type GlyRs. In addition, chelating zinc with tricine did not reduce the degree of enhancement of GlyR function by ethanol as it did in wild type GlyRs. Together these findings suggest that nanomolar concentrations of zinc are necessary for modulating ethanol action the GlyR, and more specifically they suggest that the D80 position is crucial in mediating zinc/ethanol interactions at the GlyR.

Although our findings demonstrate that the D80 position is important for zinc enhancement of ethanol modulation at GlyRs, there are some limitations that could be addressed in the future with additional experiments. For example, there are other amino residues on the α1 GlyR subunit (T151, E192, D194, and H215) that are also important for the enhancing effects of zinc (Miller et al., 2005). The

effects of introducing mutations at these alternative zinc sites on ethanol modulation of GlyR function have not been tested, and therefore the possibility exists that altering any of the previously identified high-affinity zinc binding sites on the GlyR could produce results similar to those we observed in mutant D80A GlyRs, and therefore zinc/ethanol interactions may not be specific to the D80 position. In addition, the possibility cannot be excluded that there are additional sites on the GlyR that are important for zinc enhancement of function, but that have not yet been identified. Finally, other studies of zinc action at ligand-gated ion channels have suggested tetradentate coordination between zinc and the protein (Trudell et al., 2008). The high-affinity zinc sites that were not included in this study (T151, E192, D194, and H215) are proposed to contribute to a zinc-binding pocket (Miller et al., 2005) and are consistent with the idea of zinc binding to the receptor in a tetradentate manner. However, there are no residues near D80 that are postulated to similarly participate in the coordination of zinc binding in this region of the GlyR (Laube et al., 2000).

Another caveat of zinc-dependent ethanol modulation of GlyR function is that zinc exerts biphasic effects on GlyR function. Basal levels of tonic zinc and synaptic zinc are estimated in the nanomolar to low micromolar range (Frederickson et al., 2006; but there are neurological events in which brain zinc levels exceed 10 μM and zinc levels in this range inhibit GlyR function via action at several sites (H107, H109, T112, and T133) with lower affinity for zinc binding. Neither the effects of higher (micromolar) zinc concentrations nor the effects of introducing mutations at any of these low affinity sites on ethanol action at the GlyR have been studied. This is potentially an important future direction

for future studies of zinc/ethanol interactions at the GlyR especially in light of our data in Chapter Three. We tested the effects of adding a series of enhancing zinc concentrations (100 nM, 500 nM, and 1 μM) on ethanol modulation of wild type α1 GlyRs, and our results suggested that ethanol-bound GlyRs may display left-shifted zinc curves such the ethanol effects were largest in the presence of 100 nM zinc and decreased with each of the two higher zinc concentrations. In the future, investigations of the effects of a wider range of zinc concentrations and other known zinc binding positions could provide valuable insights into zinc modulation of ethanol action.

The findings from this study provide evidence that zinc is critical in determining the magnitude of ethanol's effects at GlyRs. However, this is not an exclusive example of ethanol and divalent cations acting to cooperatively modulate ion channel function. For example, zinc chelation reduces the action of ethanol at NMDA receptors (Woodward & Smothers, 2003), and adding zinc increases the effects of ethanol action at NMDA receptors (Chandler et al., 1994). However, Chu et al. (1995) reported that zinc has no effect on ethanol modulation of NMDA receptor function.

In addition, magnesium enhances ethanol inhibition of NMDA receptors (Jin et al., 2008). Furthermore, there are proteins other than ion channels at which ethanol action is determined by zinc. Notably, alcohol dehydrogenase requires the co-binding of zinc with ethanol to catalyze enzymatic activity (Kang et al., 2012).

In addition to zinc, there are other known endogenous agents that modulate GlyR function. These include G-protein βγ subunits (Yevenes et al.,

2003), which are suggested in addition to G-protein α(s) to be important for ethanol modulation of GlyR function (Yevenes et al., 2008; Guzman et al., 2009; Yevenes et al., 2010; 2011a). Other endogenous modulators of GlyR function include prostaglandin E2 (selective for α3 GlyRs) and endocannabinoids (Harvey et al., 2004; Yevenes et al., 2011b). Recent evidence also suggests that cannabinoids produce analgesic effects due to action at α3 GlyRs (Xiong et al., 2011). Notably, neither prostaglandins nor endocannabinoids have been studied in combination with ethanol.

The third aim of this study was to evaluate the effects of a zinc-insensitive GlyR mutation on alcohol consumption and other related behavioral tests in mice. Heterozygous D80A KI mice served as an animal model that allowed us to expand our findings from *in vitro* experiments of recombinant α1D80A GlyRs to alcohol-related behavioral tests *in vivo*. From these experiments, a few distinct differences were observed between mutant KI and wild type mice. First, D80A KI mice had decreased consumption of and preference for ethanol compared to their littermate controls. However, this effect was only observed in females as the males showed no difference, but is consistent with the sex-differences also observed in reduced alcohol consumption and preference for other GlyR KI mice previously studied (Findlay et al., 2003; 2005; Blednov et al., 2012). Furthermore, our finding is also consistent with genetic studies that reveal a correlation between levels of hippocampal zinc and alcohol consumption in female, but not in male mice (Jones et al., 2008). However, it is difficult to interpret whether the behavioral changes observed in the D80A KI mice are due

to a general impairment in GlyR function, a decrease in ethanol modulation, a decrease in zinc signaling, or some combination of these factors.

Additional behavioral differences between D80A KI and wild type mice include LORR induced by pentobarbital, flurazepam, and ketamine. Both female and male mutant mice had increased sleep times following injections of the above drugs. This effect also is consistent with the results of behavioral tests in other GlyR mutant mice (Findlay et al., 2003; Blednov et al., 2012), suggesting that impairments in normal GlyR function potentially lead to compensatory changes in the receptors of other neurotransmitter systems such as GABA or glutamate. Although we did not quantitate changes in GlyR expression levels in wild type versus D80A KI mice in this study, findings from the initial characterizations of mice homozygous for the mutation did not show differences in strychnine binding assays performed on tissue from mutant D80A and wild type mice suggesting that mice with the D80A mutation do not contain increased or decreased numbers of GlyRs (Hirzel et al., 2006). Because changes in LORR induced by pentobarbital, flurazepam, and ketamine are a common behavioral characteristic shared by all GlyR KI mice that have been tested (Findlay et al., 2003; Blednov et al., 2012), it seems that the changes in sleep time is perhaps a likely consequence of general impairments in GlyR function as opposed to specific behavioral effects of any particular mutation.

Although there are no recent reports of brain zinc levels in human alcoholics, earlier attempts to study zinc levels in chronic heavy drinkers suggested that alcoholism is associated with low serum and brain levels of zinc (Mezano & Carlen, 1994). However, there are conflicting accounts in which

some cite low levels of brain zinc in alcoholics, whereas others report no differences between alcoholics and controls (Khan et al., 1984). In these early reports, there are confounds due to methodical limitations, but also confounds often associated with postmortem alcoholic tissue such as poly drug use and comorbid conditions. Despite this, the initial low brain zinc hypothesis in alcoholism is in agreement with more recent genetic studies in mice that found an inverse relationship between hippocampal zinc levels and alcohol consumption (Jones et al., 2008). In recent years, newer quantification methods for measuring unbound versus protein bound zinc have enabled studies to quantify tonic levels of free zinc in cerebrospinal fluid, whereas previously used methodologies strictly measured total or protein bound zinc (Frederickson et al., 2006). By using newer technologies, it's possible that the early hypothesis that alcoholics contain low levels of brain zinc could be revisited such that doing so may resolve the formerly conflicting findings.

The importance of zinc signaling at GlyRs for normal neurological function was described in earlier chapters in the context of the impairments of homozygous and heterozygous D80A KI mice. However, the significance of zinc homeostasis for proper brain function extends beyond the scope of this study and is more broadly evident in a large list of disorders with which zinc dysregulation can be associated. These include Parkinson's, Alzheimer's, schizophrenia, attention deficit and hyperactivity disorder, depression, amyotrophic lateral sclerosis, Down's syndrome, multiple sclerosis, Wilson's disease, and Pick's disease. In addition, more transient alterations in brain zinc have been described in epilepsy, traumatic brain injury, and even alcoholism (Grabrucker et al., 2011).

Accordingly, the development of zinc-related pharmacotherapies could have a widespread impact.

Overall, the findings from this study demonstrate that the effects of ethanol at GlyRs (α1, α2 and α3) are zinc-dependent, and the implications of these findings are multi-faceted. For example, future studies of ethanol action at recombinant GlyRs should account for contaminating levels of zinc as they are suffice for affecting modulation by ethanol. In addition, our data highlight that understanding zinc/ethanol interactions is necessary in ultimately defining the mechanism of action of ethanol at the GlyR. Furthermore, findings from our behavioral tests in GlyR KI mice containing the zinc-insensitive D80A mutation indicate that normal GlyR function and zinc signaling are important for alcohol consumption and other related behavioral tests. Ultimately, by better understanding the sites and mechanisms by which ethanol produces its intoxicating effects, improved pharmacotherapies and other treatment options can be developed for the treatment of alcohol-related disorders.

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